Characterization of the Achromobactin Iron Acquisition Operon in *Sodalis glossinidius*

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Sodalis glossinidius is a facultative, extra- and intracellular symbiont found in most tissues of the tsetse fly (*Glossinia* sp.). *Sodalis* has a putative achromobactin siderophore iron acquisition system on the pSG1 plasmid. Reverse transcription (RT)-PCR analysis revealed that the achromobactin operon is transcribed as a single polycistronic molecule and is expressed when *Sodalis* is within the tsetse fly. Expression of the achromobactin operon was repressed under iron-replete conditions; in a mutant that lacks the iron-responsive transcriptional repressor protein Fur, expression was aberrantly derepressed under these iron-replete conditions, indicating that the Fur protein repressed achromobactin gene expression when iron was plentiful. A putative Fur binding site within the *Sodalis* achromobactin promoter bound Fur in *Escherichia coli* Fur titration assays. Wild-type *Sodalis* produced detectable siderophore *in vitro*, but a mutation in the putative achromobactin biosynthesis gene *acsD* eliminated detectable siderophore production in *Sodalis*. Reduced growth of the siderophore synthesis mutant was reconstituted by addition of exogenous achromobactin, suggesting the strain retains a functional siderophore transport system; however, reduced growth of a *Sodalis* ferric-siderophore outer membrane receptor mutant with a mutation in *acr* was not reconstituted by exogenous siderophore due to its defective transporter. The *Sodalis* siderophore synthesis mutant showed reduced growth in tsetse that lacked endogenous symbionts (aposymbiotic) when the flies were inoculated with *Sodalis* intrathoracically, but not when inoculated *per os*. Our findings suggest that *Sodalis* siderophores play a role in iron acquisition in certain tsetse fly tissues and provide evidence for the regulation of iron acquisition mechanisms in insect symbionts.

Many arthropods harbor bacterial symbionts that must acquire nutrients for themselves and provide nutrients or other advantages to their hosts (for a review, see reference 1). The tsetse fly (*Glossina morsitans morsitans*), the sole vector of the human and animal trypanosomes (*Trypanosoma brucei* subsp.) in sub-Saharan Africa, harbors three bacterial symbionts: *Sodalis glossinidius*, *Wigglesworthia glossinidia*, and *Wolbachia* sp. (reviewed in reference 2). Both *Sodalis* and *Wigglesworthia* are passed vertically through tsetse populations via maternal milk gland secretions (3, 4). *Wigglesworthia* is found intracellularly within an organ immediately adjacent to the tsetse's midgut called the bacteriome, as well as extracellularly within maternal milk gland secretions (3). This obligate symbiont presumably supplies the tsetse with supplemental vitamins and cofactors missing from its vertebrate host blood-specific diet (5).

In contrast to *Wigglesworthia*, less is known about the role of commensal *Sodalis* in tsetse biology. *Sodalis* is a facultative intra- and extracellular symbiont that resides in the midgut, hemolymph, milk glands, salivary glands, muscle, and fat body tissues of some tsetse fly species (6–9). The bacterium likely provides some benefit to tsetse, as flies exhibit a reduced life span when *Sodalis* is selectively eliminated via treatment with antibiotics (10). Additionally, when *Sodalis* is selectively eliminated from tsetse, the fly is more refractory to infection with trypanosomes (10). This finding suggests that *Sodalis* modulates its host's immune response against the pathogen.

Sodalis, like other bacteria that live within eukaryotic hosts, must acquire the essential element iron in order to survive and proliferate within the tsetse fly. In blood-feeding insects, iron is a component of blood meal hemoglobin and is sequestered within host storage molecules, including heme, transferrin, ferritin, and iron-containing proteins (11–13). *Sodalis* has several

putative iron acquisition systems, including a siderophore system that synthesizes and transports the siderophore achromobactin (14, 15). Siderophores are iron-chelating molecules secreted by microbes. After capturing iron, the iron-bound siderophores (ferrisiderophores) are taken into the bacterial cell via siderophore-specific receptors (for a review, see reference 16). *Sodalis* has 12 genes on one of its plasmids (designated pSG1) that, based on homology with achromobactin genes in other organisms, are annotated to encode an achromobactin siderophore system (15, 17).

Bacteria living within blood-feeding insects are likely exposed to large fluctuations in the amount of heme and iron present in the environment. Because extreme iron concentrations at both ends of the spectrum are detrimental to bacterial growth, expression of iron acquisition systems in bacteria that reside in blood-feeding insects is likely to be tightly regulated. Many bacteria use the conserved Fur transcriptional repressor for regulation of the expression of their iron uptake systems. The Fur repressor protein, when bound to iron, binds specific DNA sequences that generally overlap the RNA polymerase binding site in Fur-regulated promoters. Thus, expression of iron acquisition systems is repressed when sufficient iron is present in the cell (18). Although *Sodalis* was initially reported to lack the *fur* gene (17), further annotation of the genome indicated that the commensal bacterium does contain

Received 27 December 2012 Accepted 14 February 2013

Published ahead of print 22 February 2013

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doi:10.1128/AEM.03959-12

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Bacterial (<i>E. coli</i>) strains		
DH5 α	λ^- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(r $_K^-$ m $_K^-$) supE44 thi-1 gyrA relA1	38
H1717	aroB <i>fluF</i> :: λ placMu	39
BW25113	Δ (araD-araB)567 Δ lacZ4787(::rrnB-3), λ^- rph-1 Δ (rhaD-rhaB)568 hsdR514	40
JW0669-2	F $^-$ Δ (araD-araB)567 Δ lacZ4787(::rrnB-3) Δ fur-731::kan λ^- rph-1 Δ (rhaD-rhaB)568 hsdR514	41
<i>Sodalis</i> strains		
SOD	<i>S. glossinidius</i> from <i>Glossina moristans moristans</i>	S. Aksoy
SOD/pAR1219	Parent strain for <i>Sodalis</i> mutations	14
URSOD6	<i>fur</i> 183::kan pAR1219	14
URSOD8	<i>acsD</i> 1246::kan pAR1219	This study
URSOD10	<i>acr60</i> ::kan pAR1219	This study
Plasmids		
pAR1219	T7 polymerase under the control of lac UV5 promoter for inducing intron mutagenesis	42
pLR29	Promoterless GFP vector	25
pBluescript KS	High-copy-number cloning vector	43
pCS2	<i>ach</i> promoter- <i>gfp</i> on pLR29	This study
pCS9	<i>ach</i> promoter in pBKS	This study
pCS10	<i>ach</i> promoter putative Fur site 1 in pBKS	This study
pCS11	<i>ach</i> promoter putative Fur site 2 in pBKS	This study
pCS12	<i>ach</i> promoter putative Fur site 3 in pBKS	This study
pRJ19	<i>Sodalis</i> insulinase promoter-luciferase gene	This study

a functional *fur* gene whose product regulates the expression of putative heme and Sit iron acquisition systems (14).

The purpose of this study was to investigate the regulation, function, and importance of achromobactin genes in *Sodalis* biology. To do this, expression of the *Sodalis* achromobactin operon was studied *in vitro* and *in vivo*. Additionally, *Sodalis* strains with mutations in the putative synthesis and transport genes of the achromobactin operon were generated, and these strains were utilized to investigate the importance of the iron uptake system to *Sodalis* growth *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) or on Luria-Bertani agar (L agar) plates. *E. coli* strains containing green fluorescent protein (GFP) were grown in low-salt LB or on low-salt L agar (which contains 5 g of NaCl per liter). Liquid cultures were incubated at 37°C with 200 rpm aeration. *Sodalis* was grown at 25°C and 10% CO₂ on brain heart infusion (BHI) agar with 10% horse blood (Hemostat Laboratories, Dixon, CA). Liquid cultures were started in petri dishes at optical densities at 600 nm (OD₆₀₀) of 0.02 to 0.08 and incubated without aeration. Specific growth conditions for each experiment are indicated in the figure legends.

To create iron-limited conditions, 16 μ M of the iron chelator ethylene diamino-*o*-dihydroxyphenyl acetic acid (EDDA) was added to growth media (19). To create iron-replete conditions, 40 μ M FeSO₄ was added to growth media. To obtain spent medium as a source of siderophore, *Sodalis* bacteria were subcultured from 3-day-old liquid cultures into BHI containing 16 μ M EDDA and grown overnight. The culture was centrifuged at 6,000 $\times$ g, and the supernatants were then sterilized by passage through a 0.2- μ m filter.

Antibiotics were used for *E. coli* at the following concentrations: carbenicillin (Carb), 125 μ g/ml; ampicillin (Amp), 50 μ g/ml; chloramphenicol (Cam), 30 μ g/ml; kanamycin (Kan), 50 μ g/ml; and tetracycline (Tet), 12.5 μ g/ml. Antibiotics were used for *Sodalis* at the following concentrations: Carb, 125 μ g/ml; Amp, 20 μ g/ml; Cam, 3 μ g/ml; Kan, 25 μ g/ml; and Tet, 3 μ g/ml.

Insect maintenance. *G. morsitans morsitans* flies were maintained in Yale's insectary at 24°C with 50 to 55% relative humidity. All flies received defibrinated bovine blood (Hemostat Laboratories) every 48 h through an artificial-membrane feeding system (20). To generate aposymbiotic flies, pregnant, wild-type female tsetse were fed a diet supplemented with tetracycline (20 μ g/ml of blood) to clear all endogenous microbes and yeast extract (1% [wt/vol]) to rescue the sterile phenotype associated with the absence of obligate *Wigglesworthia* (21). Offspring from these mothers, which lacked all symbiotic bacteria throughout immature development and adulthood, are here referred to as "aposymbiotic" (*Gmm*^{Ap}).

Plasmid constructions. Primer sequences for construction of the pCS series plasmids with the entire achromobactin promoter or fragments of the promoter are listed in Table 2. The 623-bp region of DNA between *acsF* (SGP1_0046) and SGP1_0047 was named the achromobactin (*ach*) promoter and was amplified using *Pfu* polymerase (Agilent, Santa Clara, CA) and primers UR337 and UR338. Three individual putative Fur binding sites identified by Virtual Footprint version 3.0 (<http://prodoric.tu-bs.de/vfp/>) (22) were amplified using primers UR407 and UR408 to give an 83-bp amplicon for site 1, UR337 and UR409 to give a 337-bp amplicon for site 2, and UR410 and UR338 to give a 235-bp amplicon for site 3. Plasmids were isolated using the QIAprep Spin Miniprep Kit and protocol (Qiagen, Valencia, CA). The plasmids pLR29 (7.2 kb) and pBluescript KS (pBKS) (2.9 kb) were digested overnight with BamHI and XbaI (Promega, Madison, WI). The linearized plasmid was purified from a 0.7% agarose gel using the QIAquick gel extraction kit (Qiagen) and ligated with the appropriate insert using T4 DNA ligase to generate the plasmids in Table 1.

pRJ19 was constructed to create a tetracycline-resistant variant of the pIL plasmid, which harbors the firefly luciferase gene under the transcriptional control of the *Sodalis* insulinase promoter (23). The tetracycline resistance gene was PCR amplified from pACYC184 (24) using primers UR005 and UR006 and PfuTurbo polymerase (Agilent Technologies, Santa Clara, CA), purified from a 0.7% agarose gel using the QIAquick gel extraction kit (Qiagen), and ligated into pIL linearized with SspI.

***Sodalis* achromobactin operon expression studies using RT-PCR.** *Sodalis* cultures were grown in BHI from a starting OD₆₀₀ of 0.02 for 3 days and then subcultured into BHI supplemented with 40 μ M FeSO₄ or 16 μ M EDDA and grown overnight. RNA was isolated from *Sodalis*

TABLE 2 Primers used in this study

Primer pair	Sequence	DNA amplified or role
SGP1_00451F SGP1_00451R	5' CTCTCCAAGCAGGATCTGTG 5' TCCACCGTCACGCTATTACTC	<i>acr</i>
SodrplB1F SodrplB1R	5' TGCTGGAACTCTCAGCAAAT 5' CTCCAGACGTTCTACCACTGC	<i>rplB</i>
UR005 UR006	5' CCCACCGGTCAAATGTAGCACCTGAAGTC 5' CCCACCGGTAACCAGTAAGGCAACCCCG	<i>tet^R</i>
UR301 UR302	5' GCCAATACCGGCCCCAGCAG 5' GCGCAGATACTGGCCGGGTG	<i>acsD</i>
UR303 UR304	5' ACCTCCAGATCGACCGGGGC 5' ACTCCCGGGCCTACGACCTT	<i>acr</i>
UR317 UR318	5' TGTGTGGTTTGCGCCGTGGT 5' GGCATTGCAAGGTGTGCCGC	<i>cbrA</i>
UR335 UR336	5' GGCAGATGCGCTGGGCAGTT 5' CGTGATGTTACCAACGCCCGTT	<i>acsF</i>
UR337 UR338	5' CGGGATCCTGCCCCGTTACGGACTTCCGG 5' TGCTCTAGACCCTGAGCCCCGGCAATAGC	<i>ach</i> promoter
UR407 UR408	5' CGGGATCCAAGCCGTGACGTTTCATCCG 5' TGCTCTAGACTCAAGAAACATGAACAGCG	Putative Fur site 1 in <i>ach</i> promoter
UR409 UR337	5' TGCTCTAGAAGCCTGTTACTAACTTTGGC 5' CGGGATCCTGCCCCGTTACGGACTTCCGG	Putative Fur site 2 in <i>ach</i> promoter
UR410 UR338	5' CGGGATCCGATCTAAAAAGTTCTTTGCC 5' TGCTCTAGACCCTGAGCCCCGGCAATAGC	Putative Fur site 3 in <i>ach</i> promoter
UR448	5' GCAAAAAGTTCAGCACCTCG	For reverse transcription of achromobactin mRNA

cultures or *Sodalis*-containing flies using the RNeasy Mini Kit (Qiagen). Reverse transcription (RT)-PCR of 10 ng of RNA was done in two steps. First, cDNA was generated using Superscript III (Invitrogen) and either random hexamer primers or RT primer UR446 in *cbrD* (SGP1_0035). Then, PCRs were carried out using GoTaq polymerase (Promega) and the following primer sets: UR335 and UR336 to amplify *acsF*, SGP1_00451F and SGP1_00451R to amplify *acr* (SGP1_0045), UR301 and UR302 to amplify *acsD*, and UR317 and UR318 to amplify *cbrA*. PCR products were run on 1 to 2% agarose gels, and the DNA was visualized with ethidium bromide staining.

In vivo expression analyses of the *Sodalis* achromobactin operon. Teneral tsetse flies were sacrificed at the teneral life stage (i.e., newly enclosed adults prior to blood meal consumption) and 96 h following feeding. Total RNA was isolated from individual tsetse flies using TRIzol (Invitrogen) and treated with RNase free-DNase I (Invitrogen). cDNA was generated from 1,000 ng of RNA from teneral flies and 5,000 ng of RNA from adult flies using Superscript II (Invitrogen) and random hexamer primers. The *acr* (achromobactin outer membrane receptor) or *rplB* (50S ribosomal subunit protein L constitutive control) sequences (197 and 148 bp, respectively) were amplified from the cDNA using GoTaq polymerase (Promega, Madison, WI) and the primers SGP1_00451F and SGP1_00451R to amplify *acr* or SodrplB1F and SodrplB1R to amplify *rplB*. The PCR products were run on 2% agarose gels, and the DNA was visualized with ethidium bromide staining.

***Sodalis* achromobactin operon expression studies using *gfp* reporter fusions.** To investigate expression of the achromobactin operon in response to environmental conditions, *E. coli* bacteria containing pLR29-

derived plasmids were subcultured 1:50 into BHI containing 40 μ M FeSO₄ or 1 to 16 μ g/ml of the iron chelator EDDA and grown either at 37°C for 5.5 to 6 h or at 25°C in 10% CO₂ for 4 h. *Sodalis* bacteria containing the appropriate plasmids were swabbed from BHI plus 10% horse blood agar plates into BHI at an OD₆₀₀ of 0.04 with the same FeSO₄ or EDDA additions described above for *E. coli*. The *Sodalis* cultures were incubated at 25°C in 10% CO₂ for 2 days. After the incubation period, cells were fixed in 2% paraformaldehyde (25). A FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ) fluorescence-activated cell sorter with excitation at 488 nm was used to measure sample fluorescence with the following settings: forward scatter (FSC), E01; side scatter (SSC), 505; and relative fluorescence between 515 and 545 nm (FL1), 798.

β -Galactosidase assays. Strains were grown overnight at 37°C with aeration at 200 rpm in LB with 16 μ g/ml of the iron chelator EDDA (iron limited) or with 25 μ M iron sulfate (iron replete). β -Galactosidase assays were done as described by Miller (26).

Construction of *Sodalis* mutants. The *acsD* and *acr* mutants were constructed using the Targetron Intron Mutagenesis kit (Sigma-Aldridge, St. Louis, MO) as described previously (14). Briefly, the group II intron on pACD4K-C-loxP was altered according to the manufacturer's instructions to contain an *acsD* or *acr* targeting site located 1,246 bp and 60 bp 3' of the *acsD* and *acr* start codons, respectively. The altered intron plasmids (pRJ20 for *acsD* and pRJ22 for *acr*) were electroporated into *Sodalis* (27). The electroporation mixtures were transferred to 5 ml of BHI and incubated overnight. After overnight incubation, the samples were centrifuged (13,000 $\times$ g for 1 min), and the pellets were resuspended in 3 ml BHI containing chloramphenicol and 1% glucose and incubated overnight.

Intron expression was induced with 500 μ M IPTG for 1 h. The IPTG-treated sample was centrifuged ($13,000 \times g$ for 1 min), and the pellet was resuspended in 1 ml BHI broth containing 1% glucose and incubated for 1 h. The sample was centrifuged ($13,000 \times g$ for 1 min), and the pellet was resuspended in 400 μ l BHI broth and spread on four BHI agar plates containing 10% horse blood and kanamycin. Single colonies were restreaked after approximately 1 week on BHI agar plates containing 10% horse blood and kanamycin. Insertion of the intron into the *Sodalis acsD* or *acr* gene and elimination of the wild-type gene were confirmed by PCR analysis using the *Sodalis* primer set UR301/UR302 for *acsD* or UR303/UR304 for *acr*, which flank the gene to be knocked out.

Siderophore assays. To measure siderophore production, the chrome-azurol S (CAS) universal siderophore chemical assay was used as described by Schwyn and Neilands (28). Briefly, CAS agar was prepared with 0.15 M NaOH, 0.1 M PIPES buffer, MM9 salts ($10\times$; 3 g/liter KH_2PO_4 , 5 g/liter NaCl, 10 g/liter NH_4Cl) from a $10\times$ base, and 1.5% Bacto agar. After autoclaving the media, the following supplements were added to the indicated final concentrations: 0.6% deferrated Casamino Acids, 0.4% glucose, 1 mM MgSO_4 , 1 mM CaCl_2 , 0.1 mM thiamine monophosphate, 15.1 μ M NAD, and 10 ml chrome-azurol S-HDTMA [hexadecyl(trimethyl)azanium]. *Sodalis* strains were grown in BHI for 2 to 3 days from an OD_{600} of 0.04, and 16 μ g/ml EDDA was added 1 day before plating to induce siderophore expression. Bacteria (6.5×10^7 to 1.7×10^8) were centrifuged, and the resulting pellets were resuspended in 100 μ l BHI and spotted onto a CAS agar plate. The plates were incubated at 25°C and 10% CO_2 for 5 to 7 days.

Tsetse systemic colonization assay. To assess the relationship between *Sodalis* siderophore genes and the bacterium's ability to colonize its tsetse host, 2-day-old *Gmm*^{Apo} adults were divided into two groups, each of which was injected intrathoracically with 10^3 CFU of either *Sodalis* SOD/pAR1219 or *Sodalis* URSOD8, both containing the constitutive luciferase gene on pRJ19. The flies were fed a blood meal 1 day preinjection and 2 days postinjection. At 6 days postinjection, 6 to 12 flies from each infection group were individually homogenized and assayed for luciferase activity. The number of *Sodalis* bacteria per fly was calculated by dividing the number of relative luciferase units (RLU) per fly by the number of RLU per bacterium for that strain, and the mean for each strain was reported. Statistical analyses of the data were performed using the one-way analysis of variance (ANOVA) statistics package in Microsoft Excel (Microsoft Corporation, Redmond, WA).

Tsetse per os colonization assay. To assess the relationship between *Sodalis* siderophore genes and the bacterium's ability to colonize its tsetse host, newly emerged *Gmm*^{Apo} adults were divided into two distinct groups ($n = 100$ per group), each of which was administered a meal containing 500 CFU of either SOD/pAR1219 or URSOD8 per ml of blood. Following per os inoculation with bacteria (29), the flies were maintained on heat-inactivated blood every 48 h. Gut tissue from flies of each group was microscopically dissected at 1, 3, 5, and 10 days postinoculation. With the exception of the first time point examined (day 1), all guts were harvested 2 days after the administration of a blood meal. The remaining flies were maintained for 20 additional days, at which time their guts were removed either 2 or 5 days after their last blood meal. The *Sodalis* density per tsetse gut was determined using a previously developed bacterial plating assay (29). All per os colonization assays were performed in duplicate. Tsetse sample sizes per time point are indicated in the corresponding figure legends.

Sampling and DNA extractions for detection of *acr*. *G. morsitans morsitans*, *G. fuscipes fuscipes*, and *G. pallidipes* flies were trapped in Murchison Falls, Uganda. Flies were dissected under sterile conditions, and the samples were preserved in ethanol until they were used for DNA extraction. For each individual, total DNA was prepared from whole flies using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies). The individual DNA pellets were resuspended in 100 μ l; 0.5 μ l of a 1:10 dilution was used in a 20- μ l PCR with primers SGP1_00451F and SGP1_00451R (Table 2) to detect the *acr* gene.

RESULTS

The *Sodalis* achromobactin genes are transcribed as a single operon in tsetse flies. The putative synthesis and transport genes of the achromobactin operon are interspersed with one another on plasmid pSG1 (15); therefore, these genes are likely transcribed together (Fig. 1A). To determine whether the putative achromobactin genes are transcribed as a single polycistronic RNA molecule, RT-PCR was performed using an RT primer to the last gene in the putative achromobactin operon (*cbrD*), and then the resultant cDNA was examined for the presence of the gene furthest upstream (*acsF*) and for a gene in the middle of the operon (*cbrA*) by PCR. We found that the RT reaction with the *cbrD* primer generated a cDNA that subsequently produced PCR products for *acsF* and *cbrA*, indicating that all of the achromobactin operon genes are transcribed on the same RNA molecule (Fig. 1B).

To examine whether the achromobactin operon is expressed when *Sodalis* is within the tsetse fly, we performed RT-PCR on total RNA from teneral (newly emerged, unfed) and 96-h-old flies (Fig. 1C). Isolation of intact bacterial cDNA from the RNA was verified by amplification of cDNA corresponding to the constitutive control gene *rplB* in all samples. *acr* expression was detected in both teneral and mature adult flies, suggesting a potential role for the operon in *Sodalis* within its tsetse host.

Expression of the *Sodalis* achromobactin operon is induced by limited iron availability. In our semiquantitative RT-PCR experiments, cDNA from *Sodalis* grown in the presence of an iron chelator, EDDA, produced more *acsF* and *cbrB* PCR products than cDNA from *Sodalis* grown under iron-replete conditions (Fig. 1B). These data suggested that expression of the achromobactin operon may be upregulated in response to low-iron conditions. To test this hypothesis further, the putative promoter sequence preceding *acsF* (the achromobactin [*ach*] promoter) was fused to the promoterless *gfp* gene in pLR29 to create plasmid pCS2, which was transformed into *E. coli* and *Sodalis*. The resulting strains were grown in media containing increasing concentrations of the iron chelator EDDA to lower the environmental iron concentration. GFP analysis by flow cytometry of these cultures revealed that expression from the *ach* promoter increased as iron levels decreased (Fig. 2). Expression from the *Sodalis ach* promoter, as measured by GFP levels, was 4-fold higher in *Sodalis* than in *E. coli*, but in both species expression from the *Sodalis ach* promoter increased rapidly beginning at 6 μ g/ml EDDA and reached an ~ 6 - to 9-fold increase of expression at 16 μ g/ml EDDA relative to the samples without EDDA (Fig. 2). Furthermore, to confirm that the increased expression from the *Sodalis ach* promoter at 16 μ g/ml EDDA was due to chelation of iron by EDDA, we added iron back to the EDDA-treated culture and found that GFP expression was now reduced to a level (18.9 ± 0.83) similar to that seen in the iron-replete cultures (19.83 ± 3.4).

The *Sodalis* achromobactin operon is regulated by the Fur repressor. In *E. coli*, many genes whose expression is regulated by iron availability are repressed by the Fur repressor under iron-replete conditions. *Sodalis* contains a functional Fur gene that regulates expression of *hemT* and *sitA* (14). To determine the role of the Fur protein in regulation of the achromobactin operon, RT-PCR was carried out on RNA isolated from a *Sodalis fur* mutant (URSOD6) and the corresponding parental strain (SOD/pAR1219) that were both grown under low- and high-iron conditions. In *Sodalis* containing the Fur protein, transcription of *acr*

FIG 1 *Sodalis* achromobactin synthesis and transport genes are located in an operon. (A) Putative achromobactin synthesis genes are shown in dark gray and transport genes in light gray. The sequences of putative Fur binding sites are in boldface, and the translational start site for *acsF* is underlined. (B) Starter cultures of *Sodalis* were subcultured to an OD₆₀₀ between 0.02 and 0.04 in BHI broth containing either 40 μ M FeSO₄ (Fe) or 16 μ g/ml of the iron chelator EDDA (E) and incubated for 24 h at 25°C in 10% CO₂. RNA was isolated from each sample and used to generate cDNAs with a *cbrD*-specific primer. The cDNAs were amplified using semiquantitative PCR with *acsF* or *cbrA* primers; 5 μ l of each PCR mixture was loaded onto a 2% agarose gel, and the gel was stained with ethidium bromide. (C) RNA was isolated from either teneral tsetse flies (newly eclosed and unfed) or from flies 96 h after a blood meal. The RNAs were used to generate cDNAs with random hexamers, and the cDNAs were subsequently amplified by semiquantitative PCR with either *acr* or *rplB* (constitutive control) primers. TF, teneral female; TM, teneral male; 96h F, 96-h-old female; 96h M, 96-h-old male. Five microliters of each PCR mixture was run on a 2% agarose gel, and the gel was stained with ethidium bromide.

was repressed under iron-replete conditions. In contrast, in the Fur mutant grown under the same iron-replete conditions, *acr* transcription was not repressed, and expression levels were similar to those in iron-limiting media (Fig. 3A).

To confirm the role of the Fur repressor and to examine the kinetics of achromobactin gene regulation, *gfp* fusion experiments

FIG 2 Expression from the achromobactin promoter is iron repressed. Overnight cultures of *E. coli* DH5 α (triangles) or *Sodalis* (squares) containing the achromobactin promoter-*gfp* fusion on pCS2 were subcultured 1:25 under specific iron conditions (BHI with 125 μ g/ml carbenicillin and either 40 μ M FeSO₄ or 1 to 16 μ g/ml EDDA) and incubated at 25°C for 4 h (*E. coli*) or for 2 days (*Sodalis*). The cells were fixed with 2% paraformaldehyde and analyzed using flow cytometry. The geometric means of fluorescence for 10,000 cells are shown in triplicate with standard deviations.

were designed. The pCS2 plasmid containing the *ach* promoter fused to *gfp* was transformed into an *E. coli* Fur-null mutant, JW0699-2, and the corresponding parent strain, BW25113. The parent strain showed the expected Fur-mediated regulation profile: repression of transcription of the *ach* promoter-*gfp* fusion under high-iron conditions and increased expression of the *ach* promoter-*gfp* fusion as iron levels decreased (Fig. 3B). The Fur-null mutant, however, showed loss of repression of *ach* promoter-*gfp* expression regardless of the iron conditions (Fig. 3B).

The *Sodalis* achromobactin operon promoter has a Fur binding site. A Fur titration assay (FURTA) was used to examine whether Fur-mediated repression of the achromobactin operon was due to direct binding of the Fur repressor to the achromobactin operon promoter (30). This assay is used to test for Fur binding using a Fur-regulated promoter, *fluF*, fused to *lacZ* on the bacterial chromosome in the *E. coli* strain H1717. Under iron-replete conditions, and when Fur is functioning normally as a repressor, *lacZ* expression (and thus, β -galactosidase activity) is low. However, when a high-copy-number plasmid containing a Fur binding site is added to H1717, the Fur repressor proteins bind to the plasmid Fur sites rather than the *fluF* promoter, thus allowing expression of *lacZ*, even under iron-replete conditions. H1717 containing the *Sodalis* achromobactin promoter on pCS9 showed β -galactosidase activity under iron-replete conditions, indicating that the plasmid containing the *ach* promoter is able to bind and titrate the Fur repressor away from the *fluF*-*lacZ* fusion (Table 3).

Three putative Fur binding sites, located 620 to 627 bp (site 1), 191 to 198 bp (site 2), and 27 to 45 bp (site 3) upstream of the *acsF* putative start codon, were identified within the *Sodalis* *ach* promoter using Virtual Footprint analyses (22). To determine which of these sites bound Fur, each of the putative Fur binding sites was

FIG 3 Expression from the achromobactin promoter is iron repressed via Fur. (A) Starter cultures of the indicated strains were subcultured to an OD_{600} of 0.04 into BHI broth containing either 40 μ M $FeSO_4$ (F) or 16 μ g/ml EDDA (E) and incubated for 24 h at 25°C in 10% CO_2 . RNA was isolated from each sample and used to generate cDNAs, which were amplified using semiquantitative PCR with *acr* primers. (B) Overnight cultures of *E. coli* BW25113 (parent; black bars) and JW0669-2 (Fur-null mutant; gray bars) containing the achromobactin promoter-*gfp* fusion on pCS2 were subcultured 1:25 under specific iron conditions (BHI with 125 μ g/ml carbenicillin and either 40 μ M $FeSO_4$ or 1 to 16 μ g/ml EDDA) and incubated at 37°C with aeration at 200 rpm for 4 h. The cells were fixed with 2% paraformaldehyde and analyzed using flow cytometry. The geometric means of fluorescence for 10,000 cells are shown in triplicate with standard deviations.

cloned separately onto a high-copy-number plasmid for Fur titration analysis in *E. coli*. Only the plasmid containing site 3 was able to titrate the Fur protein away from the *fhuF* promoter (Table 3). These data indicate that site 3, but not site 1 or 2, has Fur binding ability and suggest that site 3 is likely the Fur binding site responsible for the Fur regulation of the achromobactin operon.

Siderophore production is not detected in a *Sodalis acsD* mutant in CAS assays. To determine whether the siderophore production previously reported in *Sodalis* is encoded by the pSG1 achromobactin genes, a nonpolar insertion mutation was constructed in the putative achromobactin synthesis gene *acsD* using intron mutagenesis. This procedure generated a *Sodalis* mutant strain designated URSOD8. The effect of the loss of the *acsD* gene on siderophore production was measured using CAS agar plates. SOD/pAR1219, the parent of URSOD8, consistently produced large orange halos around the bacteria on CAS agar. However, the *acsD* mutant URSOD8 produced no color change on CAS agar, indicating a lack of siderophore production detectable by CAS media and suggesting that the achromobactin operon is responsible for siderophore production in *Sodalis* under these conditions (Fig. 4).

TABLE 3 The Fur binding site in the *Sodalis ach* promoter titrates Fur away from the *fhuF-lacZ* fusion in *E. coli*

Plasmid	Characteristic	β -Galactosidase activity (Miller units) ^a in:	
		Iron-limited medium	Iron-replete medium
pBKS	No Fur binding site	2,397 $\pm$ 171	25 $\pm$ 4
pCS9	<i>ach</i> promoter	2,977 $\pm$ 369	1,686 $\pm$ 366
pCS10	Putative Fur binding site 1	1,304 $\pm$ 433	1 $\pm$ 7
pCS11	Putative Fur binding site 2	1,072 $\pm$ 251	9 $\pm$ 35
pCS12	Putative Fur binding site 3	1,015 $\pm$ 86	868 $\pm$ 405

^a H1717 containing the indicated plasmid was grown for 24 h at 37°C in LB containing carbenicillin with 16 μ g/ml of the iron chelator EDDA (iron limited) or with 25 μ M iron sulfate (iron replete). β -Galactosidase assays were done as described by Miller (26). The data presented are the means of at least three experiments and the standard deviations of the means.

***Sodalis* strains that lack an intact achromobactin system have growth defects *in vitro*.** To determine whether the *Sodalis* achromobactin system enhanced growth of the bacteria, we compared the growth of two different siderophore system mutants (a synthesis and a transport mutant) to that of the parent *Sodalis* strain. The *Sodalis* siderophore synthesis mutant URSOD8 and the Acr siderophore receptor mutant URSOD10 exhibited greatly reduced *in vitro* growth relative to the parent strain (Fig. 5).

If the growth defect of URSOD8 is caused by lack of siderophore production, then addition of siderophore to the growth medium should restore growth of the achromobactin synthesis mutant URSOD8 to parental levels, since the achromobactin transport system remains intact. The addition of increasing amounts of *Sodalis* spent medium, which served as a source of siderophore, to BHI medium increased the growth of the *Sodalis* synthesis mutant URSOD8 (Fig. 6). However, the spent medium did not increase the growth of the *acr* receptor mutant URSOD10, presumably because the siderophore in the spent medium could not be transported into the cell without the outer-membrane receptor (Fig. 6).

A *Sodalis* siderophore synthesis mutant presents a compartmentalized growth defect in tsetse flies. To test the contribution of siderophore production to the growth of *Sodalis* within the tsetse fly, mutant *Sodalis* bacteria that could not make sidero-

FIG 4 The *Sodalis acsD* mutant does not make siderophores. Cultures of SOD/pAR1219 and URSOD8 (*acsD::kan*) were grown in BHI for 3 days from an OD_{600} of 0.04, and 16 μ g/ml EDDA was added 1 day before plating. Approximately 1×10^8 bacteria were pelleted, spotted onto CAS agar, and incubated for 4 days at 25°C in 10% CO_2 .

FIG 5 Attenuated growth of *Sodalys* achromobactin operon mutants *in vitro*. Two-day-old starter cultures of *Sodalys* strains SOD/pAR1219 (circles), URSOD8 (*acsD::kan*) (diamonds), and URSOD10 (*acr::kan*) (squares) were subcultured at an OD₆₀₀ of 0.08 in BHI plus 16 μ g/ml EDDA. The cultures were grown at 25°C in 10% CO₂, and the OD₆₀₀ was measured to quantitate bacterial growth. The data presented are the means of three experiments, and the standard deviations of the means are indicated.

phores were inoculated into aposymbiotic tsetse, and the number of *Sodalys* bacteria was measured over time. If siderophore production contributes to survival or growth of *Sodalys* within the fly, then one would expect recovery of fewer siderophore mutant *Sodalys* bacteria than wild-type *Sodalys* bacteria over time. To do these experiments, a line of tsetse flies that lack all of their symbiotic microbes (here referred to as aposymbiotic, or *Gmm*^{ApO}) was generated (21). This tsetse line was used in an effort to eliminate the possibility of siderophore mutant bacterial cells using siderophores produced by their wild-type counterparts, thus reversing the mutant phenotype. We injected either the *Sodalys* *acsD* siderophore synthesis mutant strain (URSOD8) or the *acsD*⁺ parent strain (SOD/pAR1219), each containing the constitutive luciferase plasmid pRJ19 for bacterial quantification, into the thoraxes of *Gmm*^{ApO} flies. Then, we determined the number of *Sodalys* bacteria in the flies 6 days later by measuring luciferase levels and extrapolating that to the number of *Sodalys* bacteria. For both male and female tsetse, fewer *acsD* *Sodalys* mutants were recovered from

FIG 7 Colonization of adult *Gmm*^{ApO} fly thoraxes by *Sodalys* siderophore-positive (SOD/pAR1219) and mutant (URSOD8) strains. Two days after eclosion, *Gmm*^{ApO} flies were microinjected in the thorax with 10³ bacteria of each *Sodalys* strain (SOD/pAR1219 [black bars] or URSOD8 [gray bars]), both containing the constitutive luciferase gene on pRJ19. The flies were fed a blood meal 1 day preinjection and 2 days postinjection. At 6 days postinjection, 6 to 12 flies from each infection were individually homogenized and assayed for luciferase activity. The number of *Sodalys* bacteria per fly was calculated by dividing the number of RLU per fly by the number of RLU per bacterium for that strain, and the mean for each strain is reported here. *, *P* < 0.05 (ANOVA).

the flies, on average, than the *acsD*⁺ *Sodalys* strain (Fig. 7). The data were statistically significant (using ANOVA) for the male flies, but not for the female flies, although the female flies exhibited the same trend as did the males.

We next set out to determine whether *Sodalys* siderophore mutants are capable of colonizing the tsetse gut. We inoculated *Gmm*^{ApO} flies *per os* with either *acsD*⁺ (SOD/pAR1219/pRJ19) or *acsD* mutant (URSOD8/pRJ19) *Sodalys* bacteria (10³ CFU per ml of blood) and then determined the bacterial numbers over time. In contrast to what occurred in tsetse thoraxes, this experiment indicated that both siderophore-positive and -negative *Sodalys* bacteria were able to stably colonize their host's gut for up to 30 days when the flies were regularly offered blood meals (Fig. 8). Interestingly, at 30 days postinoculation, significantly more *Sodalys* siderophore mutants were present in starved tsetse hosts than in their regularly fed counterparts (Fig. 8). Taken together, the findings from our *in vivo* studies suggest that *Sodalys*' dependence on functional siderophores is related to the bacterium's specific niche within its tsetse host. Thus, *Sodalys*' nutritional physiology depends upon the host compartments it resides in—*Sodalys* appears to employ siderophore-independent mechanisms to acquire free iron when it resides in tsetse tissues (such as the gut) that contain large quantities of free heme but uses siderophore-dependent mechanisms in tissues in which heme iron is not plentiful.

Prevalence of the *acr* gene in *Sodalys* isolates from field-captured tsetse. The achromobactin operon has been identified in several tsetse fly species containing *Sodalys*, including *G. palpalis palpalis*, *G. austeni*, and *G. morsitans morsitans* (15, 17). Additionally, Darby et al. reported the presence of pSG1, the plasmid that carries the achromobactin operon, in nine of nine *Sodalys*-positive wild-trapped flies (15). We extended this analysis and examined *Sodalys*-positive tsetse flies (*G. morsitans morsitans*, *G. fuscipes fuscipes*, and *G. pallipedes*) caught in Murchison Falls, Uganda. We tested five *Sodalys*-positive wild-trapped flies for the presence of

FIG 6 The growth defect of a *Sodalys* *AcSD* mutant, but not an *Acr* mutant, can be rescued by spent medium. Two-day-old starter cultures of *Sodalys* strains in BHI containing 40 μ M FeSO₄ (with 25 μ g/ml kanamycin for mutants) were subcultured at an OD₆₀₀ of 0.02 in BHI containing either 0%, 1%, or 10% filter-sterilized spent medium obtained from wild-type *Sodalys*. The cultures were grown at 25°C in 10% CO₂ for 3 days, and the OD₆₀₀ was measured to quantitate bacterial growth. The data presented are the means of three experiments, and the standard deviations of the means are indicated.

FIG 8 Colonization of adult *Gmm*^{Δpo} fly guts by *Sodalis* siderophore-positive (SOD/pAR1219) and mutant (URSOD8) strains. Distinct groups of *Gmm*^{Δpo} flies were administered an initial heat-inactivated blood (HIB) meal inoculated with either SOD/pAR1219 or URSOD8 *Sodalis* cells, both containing the constitutive luciferase gene on pRJ19. Thereafter, all flies were maintained on HIB for the entire 30-day experiment. Every other day for 10 days, *Sodalis*-colonized *Gmm*^{Δpo} flies were sacrificed, and their gut contents were plated to determine bacterial density. The remaining flies were maintained for an additional 20 days, at which point they were sacrificed in either normal or starved (5 days without a meal) states. No significant difference in bacterial cell density was observed between siderophore-positive and -negative *Sodalis* strains in normally fed *Gmm*^{Δpo} flies. However, in comparison to the parent strain, significantly more siderophore mutants were found in 30-day-old starved *Gmm*^{Δpo} flies. Each symbol represents one fly gut. Statistical significance was determined by a Mann-Whitney test.

the putative achromobactin outer-membrane receptor gene (*acr*) by PCR. All the *Sodalis*-positive flies had the *acr* gene (Fig. 9). In combination with the previous reports, this suggested that the achromobactin operon might play a role in *Sodalis* biology in the wild.

DISCUSSION

This study was undertaken to investigate the regulation and function of the achromobactin siderophore system in the commensal tsetse fly symbiont *Sodalis glossinidius*. Our data show that the achromobactin operon is expressed by *Sodalis* *in vivo* in the tsetse fly, that the expression of this operon is regulated directly by available iron levels via the Fur repressor protein, and that it contributes to the growth of *Sodalis* under certain conditions. Although the regulation and role of siderophores has been well studied in some plant symbionts, the iron acquisition system has not been studied as extensively in animal symbionts.

Mutations were made in both putative siderophore synthesis (*acsD*) and uptake (*acr*) genes of the achromobactin operon in order to deduce the functions of the genes and the importance of the system in a biological setting. The *Sodalis acsD* gene encodes a putative achromobactin biosynthesis protein, which is 75% similar to AcsD from *Dickeya didantii* (formerly *Erwinia chrysanthemi*). Furthermore, the *Sodalis acr* gene encodes a putative achromobactin outer membrane receptor that is 78% similar to Acr from *D. dadantii*. *Sodalis* strains with a mutation in *acsD* or *acr* had growth defects *in vitro* relative to the parental strain. Other bacteria exhibit similarities to *Sodalis* in this respect. For example, a *Pseudomonas syringae* complete siderophore mutant exhibited reduced growth relative to wild-type *P. syringae*, indicating the importance of siderophore iron acquisition under low-iron conditions for this species, as well (31).

The *Sodalis acsD* mutant did not produce detectable siderophore on CAS agar plates, but its growth defect could be restored

FIG 9 Presence of the *acr* gene in *Sodalis* field isolates. DNA from *Sodalis*-positive tsetse flies (*G. morsitans morsitans* [Gmm_003 and Gmm_013], *G. fuscipes fuscipes* [Gff_071], and *G. pallipedes* [Gpd_004 and Gpd_019]) was tested for the presence of the *Sodalis* achromobactin outer-membrane receptor gene (*acr*) by PCR with *acr*-specific primers SGP1_00451F and SGP1_00451R. *Sodalis* DNA from laboratory-reared tsetse (Gmm_lab) and water were used as positive and negative controls, respectively.

by adding exogenous siderophore from spent medium in which wild-type cells were grown. This is consistent with the characterization of AcsD as encoding an essential enzyme in the biosynthetic pathway for achromobactin in the plant pathogens *D. dadantii* and *P. syringae* (31, 32). Therefore, this *Sodalis* mutant likely lost siderophore production ability due to disruption of the enzymatic pathway that produces achromobactin. Because siderophore production was completely lost with a mutation in only the achromobactin biosynthesis system, *Sodalis* likely secretes only one type of siderophore. In contrast to the *acsD* mutant, the *Sodalis acr* mutant is predicted to encode an achromobactin transporter protein, not a synthesis protein. Addition of exogenous siderophore did not restore growth of the *acr* mutant, presumably because the siderophore in the spent medium could not be transported into the cell. These data are consistent with the characterization of Acr as an essential protein for the transport of siderophore into the cell.

Within the tsetse fly host, *Sodalis* may have varying access to iron sources, depending on the location within the fly and the interval between blood meals. *Sodalis* does possess other potential iron acquisition systems in addition to the siderophore system, including a putative heme transport system that it may utilize in environments where heme is an iron source (14). Our data suggest that different iron acquisition systems may be important for *Sodalis* in different situations. RT-PCR analysis showed that the *acr* gene is expressed when *Sodalis* is in the fly, indicating a biological role for the achromobactin operon. We found that *Sodalis* mutants that lacked the ability to make achromobactin were attenuated for growth when inoculated into the tsetse hemocoel, but not when inoculated *per os* into the fly's gut. In an effort to reduce vertebrate complement-mediated killing of *Sodalis*, blood is heat inactivated prior to the addition of *Sodalis* cells and subsequent administration to tsetse (29). This process lyses the red blood cells, thereby releasing hemoglobin, heme, and/or large quantities of free iron as potential iron sources. Under these circumstances, *Sodalis* would no longer require achromobactin when residing in the iron-replete gut of tsetse. *Sodalis* has a putative hemolysin gene

that is expressed in the fly that could potentially help access these iron sources in the blood meal in the tsetse gut (17). In contrast, when *Sodalis* bacteria reside in more iron-limited environments in tsetse (such as the hemocoel), achromobactin-mediated iron acquisition may be beneficial.

Siderophore systems like achromobactin are important for growth of pathogenic bacteria *in vivo*. For example, in *D. dadantii*, which secretes achromobactin and chrysobactin siderophores, addition of exogenous chrysobactin within the context of the host leaf cells enhances bacterial growth, and both siderophores contribute to plant virulence. (33, 33a). In contrast, the contribution of siderophore production to the success of an animal symbiont has not been studied extensively, although there is one report of siderophore production in the nematode symbiont *Photorhabdus luminescens* (34).

Due to fluctuating iron availability within the tsetse host and the high toxicity of excess iron, regulation of the expression of energy-expensive iron uptake systems should be advantageous. Analysis of *Sodalis* RNA showed that the 12 genes of the achromobactin operon are transcribed as a single polycistronic RNA molecule. Thus, transcriptional regulation of this operon can be controlled by the single promoter upstream of the *acsF* gene (designated the *ach* promoter). The *ach* promoter was regulated in response to iron availability, with increasing expression as iron became limited (and thus the need for a siderophore system increased). Our studies suggest direct binding of the transcriptional regulator protein Fur to a site in the achromobactin promoter is responsible for repression of the promoter under iron-replete conditions, since mutants that lack Fur constitutively express *acr* and since the *Sodalis ach* promoter DNA sequence can titrate Fur away from other promoters. The fact that this regulation was also observed in *E. coli* containing the *Sodalis ach* promoter-*gfp* fusion suggests that the *Sodalis* Fur binding sites have been conserved over time and are capable of being recognized by *E. coli* Fur.

Iron regulation of siderophore systems is mediated by direct binding of Fur in bacterial pathogens, including *Yersinia pestis*, *Dickeya dadantii*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, and *Staphylococcus aureus* (31, 33, 35–37). Furthermore, two other potential *Sodalis* iron acquisition systems, the heme uptake system and the Sit system, are also Fur regulated (14). The fact that *Sodalis* has retained a functional iron-responsive regulatory protein, Fur, despite substantial genome erosion (17) suggests that the ability to regulate gene expression in response to environmental iron levels is important for the maintenance of *Sodalis* in natural tsetse fly populations.

ACKNOWLEDGMENTS

We thank the following individuals for their generous help: Erich Telleria for tsetse fly DNA samples and Dominique Durante for technical assistance.

This work was supported by Public Health Service grants AI084201 and AI094343 awarded to L.J.R.-J., by the Thomas F. and Kate Miller Jeffress Memorial Trust, and by funding from the University of Richmond School of Arts and Sciences.

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