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Elizabethkingia anophelis: Molecular Manipulation and Interactions with Mosquito Hosts

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Flavobacteria (members of the family *Flavobacteriaceae*) dominate the bacterial community in the *Anopheles* mosquito midgut. One such commensal, *Elizabethkingia anophelis*, is closely associated with *Anopheles* mosquitoes through transstadial persistence (i.e., from one life stage to the next); these and other properties favor its development for paratransgenic applications in control of malaria parasite transmission. However, the physiological requirements of *E. anophelis* have not been investigated, nor has its capacity to perpetuate despite digestion pressure in the gut been quantified. To this end, we first developed techniques for genetic manipulation of *E. anophelis*, including selectable markers, reporter systems (green fluorescent protein [GFP] and NanoLuc), and transposons that function in *E. anophelis*. A flavobacterial expression system based on the promoter P*ompA* was integrated into the *E. anophelis* chromosome and showed strong promoter activity to drive GFP and NanoLuc reporter production. Introduced, GFP-tagged *E. anophelis* associated with mosquitoes at successive developmental stages and propagated in *Anopheles gambiae* and *Anopheles stephensi* but not in *Aedes triseriatus* mosquitoes. Feeding NanoLuc-tagged cells to *A. gambiae* and *A. stephensi* in the larval stage led to infection rates of 71% and 82%, respectively. In contrast, a very low infection rate (3%) was detected in *Aedes triseriatus* mosquitoes under the same conditions. Of the initial *E. anophelis* cells provided to larvae, 23%, 71%, and 85% were digested in *A. stephensi*, *A. gambiae*, and *Aedes triseriatus*, respectively, demonstrating that *E. anophelis* adapted to various mosquito midgut environments differently. Bacterial cell growth increased up to 3-fold when arginine was supplemented in the defined medium. Furthermore, the number of NanoLuc-tagged cells in *A. stephensi* significantly increased when arginine was added to a sugar diet, showing it to be an important amino acid for *E. anophelis*. Animal erythrocytes promoted *E. anophelis* growth *in vivo* and *in vitro*, indicating that this bacterium could obtain nutrients by participating in erythrocyte lysis in the mosquito midgut.

Elizabethkingia species have attracted much interest because of their close biologic associations with *Anopheles* malaria vector and *Aedes* dengue fever vector mosquitoes (1–5). *Elizabethkingia* was detected in diverse sources of mosquitoes (field caught, seminatural reared, and insectary reared) sampled in different regions (Africa, Europe, and North America) (1, 3, 4). For instance, *Elizabethkingia* or *Elizabethkingia*-like bacteria were detected in 68% of field-caught mosquito populations collected in Cameroon (3). *E. anophelis* was isolated by Kämpfer et al. from the midgut of *Anopheles gambiae* G4 reared in an insectary as a predominant bacterial species (6). Wang et al. conducted a dynamic microbial community analysis of mosquitoes reared in seminatural microcosms (in Kenya) by using pyrosequencing methods and showed that *Elizabethkingia* spp. were more abundant in mosquitoes than in water of microcosms in which larvae were reared (1). *Elizabethkingia* spp. were frequently found to be associated with *A. gambiae* at various development stages (1). Similarly, Ngwa et al. found *E. meningoseptica* was the predominant bacterium in both larval and adult *A. stephensi* mosquitoes (7). Coon et al. also showed *Elizabethkingia* was common in all life stages in *Aedes aegypti* and *A. gambiae* (8). Based on the above observations, it is very likely that some *Elizabethkingia* species are symbionts for mosquitoes.

Paratransgenesis, a "Trojan horse" concept, entails transgenic symbionts or commensal microbes interfering with a pathogen's development inside insect vectors (3, 9, 10). It presents an alternative intervention strategy for vector-borne pathogen transmission (11). A candidate paratransgenesis agent should have the following characteristics: (i) it can be cultured *in vitro* and propagated; (ii) it can be genetically modified and introduced into female mosquitoes; (iii) it infects and stably persists in mosquitoes (12). Mosquito-associated commensal *Elizabethkingia* spp. may be excellent candidates for paratransgenesis tool development. For example, anti-*Plasmodium* activity was demonstrated in *E. meningoseptica* and *E. anophelis* in two recent studies. Ngwa et al. showed the ethyl acetate extracts of broth culture of *E. meningoseptica in vitro* had antiplasmodial activity (50% inhibitory concentration [IC_{50}], 0.25 mg/ml) against the *P. falciparum* asexual blood stages, showed antigametocidal activity, and reduced 58% of *P. falciparum* density at the IC_{50} dose (7). Bahia et al. showed that *E. anophelis* had a prominent effect on *Plasmodium* parasite development when it was introduced at a low bacterial dose (10^3 cells/μl), reducing oocyst load (13). When Akhouayri et al. injected *E. meningoseptica* into *Anopheles* mosquitoes, the bacteria were highly virulent in adults, a process related to melanotic le-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics and/or plasmid construction[a]	Reference or source
E. coli strains		
EC100D *pir*$^+$	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG pir*$^+$ (DHFR)	Epicentre
S17-1	*hsdR17*(r_K^- m_K^-) *recA RP4-2* (Tcr::Mu-Kmr::Tn*7* Str^r)	49
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Clontech
E. anophelis strains		
MSU001	Isolated from *Anopheles gambiae* at Michigan State University	This study
SCH814	Strain carrying expression cassette P*ompA*+*nluc*	This study
SCH837	Strain carrying expression cassette P*ompA*+*gfp*	This study
Plasmids		
pGEM-T Easy	Cloning vector; Amp^r	Promega
pHimarEm1	*mariner* transposon functional in flavobacteria; Km^r (Em^r)	34
pSCH760	Modified pHimarEm1 with MCS site SmaI-BamHI-SacII; Km^r (Em^r)	This study
pSCH722	P*ompA*+*nluc* on pCP29; Amp^r (Em^r)	38
pFj29	P*ompA*+*gfp* on pCP29; Amp^r (Em^r)	50
pSCH770	P*ompA*+*gfp* on pGEM-T Easy with SmaI and SacII restriction sites; Amp^r	This study
pSCH773	P*ompA*+*gfp* inserted at SmaI and SacII sites on pSCH760; Km^r (Em^r)	This study
pFD1146	Shuttle plasmid between *Bacteroides* and *E. coli*; Sp^r (Em^r)	51
pNJR5	IncQ, *E. coli-Bacteroides* shuttle vector; Km^r (Em^r)	52
pSCH791	P*ompA*+*nluc* on pGEM-T Easy; Amp^r	This study
pSCH801	P*ompA*+*nluc* on pSCH760; Km^r (Em^r)	This study

[a] Unless indicated otherwise, antibiotic resistance phenotypes are those expressed in *E. coli*. Antibiotic resistance phenotypes listed in parentheses are those expressed in *E. anophelis* strains but not in *E. coli*. DHFR, dihydrofolate reductase.

sions in fat body tissues (5). Together, these studies demonstrate that *Elizabethkingia* species impact their host mosquitoes' physiology and interactions with malaria parasites. Recently, several genomes from *E. meningoseptica* and *E. anophelis* were sequenced and annotated (2, 14, 15). A number of genes, such as those related to sugar transportation/utilization, blood cell lysis, and the antioxidative system, were found and provided insights into possible flavobacterial symbiotic relationships with mosquito hosts (2). *E. anophelis* could therefore be a good model system to investigate how predominant bacteria interact with their hosts, vectored disease agents (parasites/viruses), and other associated microbes.

Despite their wide distribution in nature, potential importance in mosquito physiology, and paratransgenesis potential, the infection range, colonization mechanism(s) in mosquitoes, and nutrient requirements of *E. anophelis* remain poorly known. No genetic tools have been available for molecular manipulation of *Elizabethkingia* spp., impairing the study of symbiotic relationships with mosquitoes and interactions with other gut microbes and parasites. Genetic manipulation of flavobacterial members has been extremely difficult because the available genetic tools that are functional for proteobacteria do not function in flavobacteria, owing to the unique transcription initiation signals (promoter sequences) in the *Bacteroidetes* (16, 17). The objectives of the present study were as follows: (i) to develop molecular tools for tracking the fate of *E. anophelis* cells in mosquitoes; (ii) to investigate the mosquito host range of *E. anophelis*; (iii) to characterize nutrient requirements for *E. anophelis* growth *in vivo* and *in vitro*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and molecular reagents used in this study are listed in Table 1. *Escherichia coli* DH5α was used for cloning. *E. coli* S17(λ *pir*) was used for conjugation. *E. coli* EC100D *pir*$^+$ was used for recovering transposon from *E. anophelis*. *E. coli* strains were routinely grown in Luria-Bertani (LB) broth (18). *Elizabethkingia* species were isolated from the mosquitoes *Anopheles stephensi* and *A. gambiae sensu stricto* kept in colonies at the insectary at Michigan State University. A primary isolate (from *A. gambiae*) used in experiments here was designated *E. anophelis* MSU001. M9 medium was slightly modified by adding yeast extract (0.5%, wt/vol) and peptone (1%, wt/vol). M9 medium, LB, or Casitone-yeast extract (CYE) medium was used for *E. anophelis* culture (18, 19). Liquid cultures were grown with shaking (ca. 200 rpm) at either 30°C (*E. anophelis*) or 37°C (*E. coli*). For solid LB medium, Bacto agar (Difco, Detroit, MI) was added to a final concentration of 20 g/liter with kanamycin (50 μg/ml) or ampicillin (100 μg/ml) added for plasmid selection in *E. coli* or erythromycin (Em) added (200 μg/ml) for transposon selection in *E. anophelis*. Various carbon sources and amino acids were added to modified M9 when growth tests were performed. Horse blood (Hemostat Lab, Dixon, CA) was supplemented in modified M9 medium to study its effect on bacterial growth.

Mosquito rearing. *Anopheles stephensi* Liston Johns Hopkins strain, *Aedes triseriatus* Say MSU strain, and *Anopheles gambiae sensu stricto* Giles KISUMU strain mosquitoes were used in this study. Mosquito adults were confined in 60- by 60- by 60-cm insect cages. Cages were held in a chamber (Percival, IA) maintained at 28°C ± 1°C (mean ± standard deviation) and 50% ± 10% relative humidity under a light/dark 12:12-h photoperiod without dawn/dusk transitions. Sucrose solution (10%) was placed in cages with wicks and reservoirs to provide carbohydrate. Sodium-heparinated bovine blood (Hemostat Lab, Dixon, CA) was fed to adult mosquitoes via an artificial membrane feeder for around 30 min, twice per week. After 2 days, mosquito eggs were collected on the wet filter paper which was then supported by a water-statured cotton ball in a petri dish. Filter papers containing eggs were transferred into plastic containers with distilled water for hatching. Either First Bite (Kyorin, Himeji, Japan) or Tetramin tropical fish food flakes (Tetra, Blacksburg, VA) were provided for *Anopheles* and *Aedes* larval mosquitoes in the first instar stage, respectively. After that stage, pet food (Purina Cat Chow; Nestlé) was given once per day.

Molecular manipulation methods. Genomic DNA was prepared using a Wizard genomic DNA purification kit (Promega, Madison, WI), and plasmid DNA was purified with the QIAprep spin miniprep kit (Qiagen, Germantown, MD). Restriction and modification enzymes were purchased from Promega (Madison, WI) or New England BioLabs (Beverly, MA). PCR amplifications were done with the Failsafe PCR system (Epicenter Technology, Madison, WI). Amplicons were separated in 0.7 to 1.0% (wt/vol) agarose gels, and DNA fragments were purified with the QIAquick gel extraction system (Qiagen). Ligation mixtures were transformed into *E. coli* cells, and transformants were plated onto LB plates with appropriate antibiotic selection. Resistant colonies were isolated and then screened for the acquisition of plasmids. All constructs were sequenced to verify structure.

The transposon pHimarEm1 was modified to introduce unique SmaI-BamHI-SacII restriction sites and to insert the reporter expression cassette P*ompA*+*gfp* (Table 1). PCR was done with pHimarEm1 DNA as the template and using forward primer Walker142 (CGCGGATCCGCGTCCCCCGGGGGACTTGACAACCACCCGACTTTGAACTACG) and reverse primer Walker143 (CGCGGATCCGCGTCCCCGCGGGGAGCTGCCGCATAACGGCTGGCAAATTGG). The amplicon was digested with BamHI, self-ligated, and transformed into *E. coli* S17(λ *pir*)(pSCH760) (Table 1). The reporter expression cassette P*ompA*+*gfp* was amplified with primers Walker146 (CCGCGGCCCAGGCTTTACACTTTATGCTTCCG) and Walker147 (CCCGGGATTATAGGGAATTCCGGACCGGTACC) and using pFj29 as the template according to standard procedures (20). The PCR product (P*ompA*+*gfp*) was first inserted into a T-easy vector (pSCH770) (Table 1). The insert was released from pSCH770 by using SmaI and SacII enzymes and inserted into the same sites on plasmid pSCH760, creating the chromosome-tagging reporter construct P*ompA*+*gfp* on pHimarEm1(pSCH773) (Table 1).

The *nluc* gene, encoding NanoLuc luciferase, was amplified with plasmid pSCH722 (Table 1) as a template by using forward primer Walker156 (ACCCGGGAACACTTAGACAAGGCAATAGAAGC) and reverse primer Walker157 (ACCGCGGTTAGACGTTGATGCGAGCTGAAGCAC) and cloned into the T-easy vector (pSCH791). The gene *nluc* was next released from pSCH791 with SmaI and SacII and inserted into the sites on pSCH760, leading to a transposon with a NanoLuc reporter (pSCH801) (Table 1). To investigate the insertion site for the transposon, genomic DNA was extracted, digested with XbaI, self-ligated, and introduced into the *E. coli*(λ *pir*) strain. The plasmid was sequenced with primers Walker85 (TGGGAATCATTTGAAGGTTGG) and Walker86 (TCGGGTATCGCTCTTGAAGGG).

For bacterial conjugation, both donor and recipient cells were cultured to mid-log phase, concentrated by centrifugation (4,500 × *g*, 15 min), washed once with LB, and resuspended in either LB (recipient cells) or a 1:1 mixture of LB and 10 mM $MgSO_4$ (donor cells). The mixture was spotted on an LB agar plate and incubated overnight at 30°C. Following incubation, the cells were scraped off the agar and resuspended in LB broth. The homogenized suspension was spread on LB plates containing 200 μg/ml of Em to select for transconjugants. Em-resistant colonies were selected and purified after 48 h of incubation at 30°C. Following the above procedures, plasmid pSCH773 or pSCH801 in *E. coli* S17(λ *pir*) was separately conjugated into *E. anophelis*, leading to the green fluorescent protein (GFP) reporter strain SCH837 or NanoLuc reporter strain SCH814, respectively (Table 1). Em-resistant transconjugants were screened by using the primers Walker140 (TTCCTTGCGCAGCTGTGCTCGAC) and Walker141 (CGCTCAGAAGAACTCGTCAAGAAG). Detection of the respective transposase gene in transconjugants was conducted using primers Walker186 (GCAAAATTCAAGCGTGGTGAAATGAGC) and Walker187 (CGAGCATCCTTTTGAGGTCTGAGAAC).

Epifluorescence microscopy. SCH837 (GFP-tagged) cells were visualized with an Olympus Provis AX70 microscope, equipped with appropriate filters, a mercury lamp for UV light source, and a DP-50 digital camera linked to an external PC. Second-instar larval mosquitoes were fed a suspension of the appropriate strains of *E. anophelis* at room temperature for 2 h, killed by 100% ethanol, transferred onto a 0.1% thin layer of agarose on the microscope slide, and observed using a UV filter.

Determination of GFP and NanoLuc reporter activity in *E. anophelis*. Quantitative analysis of GFP or NanoLuc production was performed using a SpectraMax M5 (Molecular Devices, CA) or EnVision automated microplate reader (PerkinElmer, MA). Aliquots of cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.4 and subjected to fluorescence determination in a 96-well microtiter plate (Costar, Corning, NY). GFP fluorescence was determined at an excitation wavelength of 490 nm, an emission wavelength of 530 nm, and a cutoff of 515 nm. The MSU001 strain without a *gfp* gene was used as the blank for calculation of the relative fluorescence units (17).

For determination of NanoLuc reporter activity, the cells or mosquitoes were sampled, homogenized, diluted, and immediately added to an equal volume of NanoLuc assay buffer (Promega, Madison, WI), and light intensity was quantified in 96-well microtiter plates by using a plate reader according to the manufacturer's protocol. If necessary, *E. anophelis* cells were first lysed with passive lysis buffer (PLB; Promega) and lysozyme, and then the lysate was mixed with an equal volume of NanoLuc assay buffer as described above. Standard curves were established to quantify the relationship between bacterial density and luminescence.

Analysis of digestion. Third-instar larval mosquitoes (*Aedes triseriatus*, *A. gambiae*, and *A. stephensi*) were starved for 2 h in sterile water at room temperature before incubation with reporter strain SCH814 at the log phase of growth for 2 h. Next, larvae were extensively rinsed with water and immediately transferred into a 6-well plate (four larvae in 2 ml of sterile H_2O per well). Bacteria in the larvae and the incubation solution were sampled at time points 0, 1, 1.5, 2, and 2.5 h. The four larvae in each well were pooled, homogenized with a sterile pestle, centrifuged, washed with phosphate-buffered saline (PBS), resuspended in PBS, and subjected to NanoLuc reporter analysis as described above.

Bacterial infection tests. Reporter-tagged strains (SCH814 and SCH837) were fed to larval and adult mosquitoes to track their fates. For infection tests in the larval stage, larval mosquitoes (second instar) were incubated overnight with SCH814 or SCH837 with a final concentration at ~2.4×10^8 CFU/ml, during which time larvae were actively feeding on the suspension. The exposed larvae were extensively washed and reared in 300 ml of distilled water until they molted to the pupal stage, a nonfeeding stage. Pupae were collected, washed extensively in sterile water, and placed in a container for adult mosquito emergence. Adult mosquitoes were randomly collected, processed by homogenization using a sterile pestle, and subjected to the luciferase assay. To infect adult *A. stephensi* mosquitoes, SCH814 was cultured overnight at 30°C, pelleted, washed, and adjusted to ~2.4×10^8 CFU/ml in sterile 10% sucrose solution. After feeding for 16 to 24 h, the bacterial solution was replaced with fresh sterile 10% sucrose. At specific times (days), mosquitoes were sampled randomly and subjected to the luciferase assay.

Statistical analyses. Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC).

Nucleotide sequence accession number. The GenBank accession number of 16S rRNA sequence for *E. anophelis* strain MSU001 is KP125493.

RESULTS

Phylogenetic placement of *E. anophelis* strain MSU001. Genomic DNA was extracted from strain MSU001, and the 16S rRNA gene was amplified using primers 63f and 1387r (21). Both strands of the amplified fragment were sequenced. Sequence analysis showed that it was 99% identical to the 16S rRNA genes of *E. anophelis* R26 and *E. meningoseptica*, 93% identical to that of *Riemerella anatipestifer*, and 84% identical to that of *Flavobacterium johnsoniae*. Placement of the sequence into a phylogenetic tree using MEGA (22) revealed a close relationship to several *Elizabethkingia* isolates from *Anopheles* mosquitoes (see Fig. S1 in the supplemental material).

Construction of *mariner*-based transposons carrying reporter expression cassettes. We attempted to introduce *Bacteroidetes-E. coli* shuttle plasmids (pFj29 and pFD1146) into *E. anophelis* MSU001 cells, but stable transformants were not obtained (data not shown), indicating that these plasmids were not replicable or that the host cells resisted transformation. Instead, we successfully obtained Em-resistant transformants when the mini-*mariner* transposon pHimarEm1 was conjugatively transferred into MSU001 cells, showing that introduction of foreign genetic elements was not problematic in *E. anophelis* wild-type strains. The transposon pHimarEm1 was modified with a multiple-cloning site (SmaI-BamHI-SacII) to facilitate the insertion of genes of interest (Fig. 1A). The P*ompA*+*gfp* gene expression cassette was cloned into the transposon and successfully introduced into strain *E. anophelis* MSU001; the transposition frequency was estimated at 1.5×10^{-7}. Fifty fluorescent colonies were counted using fluorimetry after being first screened from several thousand Em-resistant colonies (Fig. 1C). The colonies showed fluorescence at various intensities, ranging from 10 to 100 U/OD_{600} (Fig. 1C). The brightest-fluorescing colony (Eli28) was designated *E. anophelis* strain SCH837 and was selected for further study (Fig. 1C). SCH837 cells were readily detected, with obvious fluorescence in the foregut and midgut but much less in the hindgut of larval mosquitoes after 2 h of feeding, indicating that some of SCH837 cells were digested in *A. gambiae* (Fig. 1D). In reporter strain SCH837, a transposon carrying expression cassette P*ompA*+*gfp* was inserted in a hypothetical protein gene encoding a putative TonB-dependent receptor (WP_024563967). Similarly, in reporter strain SCH814, a transposon carrying P*ompA*+*nluc* was inserted in a hypothetical protein gene showing homology with cell envelope biogenesis protein AsmA (WP_009087586). Similar growth rates were observed in MSU001, SCH837, and SCH814 strains (see Fig. S2 in the supplemental material); thus, integration of the reporter on the *mariner* transposon in the *E. anophelis* chromosome at these sites did not affect bacterial growth. Furthermore, we validated cell growth and NanoLuc luciferase activity (see Fig. S3 in the supplemental material). The NanoLuc activity increased with cell growth during log phase (between 2 and 7 h) and remained stable in the stationary phase (between 10 and 12 h) (see Fig. S3B), indicating that expression of the *nluc* gene driven by the constitutive promoter P*ompA* was not significantly affected by growth phase (see Fig. S3C). It was therefore appropriate for estimation of cell density by determination of luciferase activity, due to the linear relationship between luciferase activity and viable cell number (see Fig. S3D).

Comparison of *E. anophelis* digestibility in mosquitoes. Ingested *E. anophelis* cells can be, alternatively, (i) preserved alive in larval mosquitoes, (ii) ejected into the surrounding solution by egestion from defecation, or (iii) digested. To track the fate after ingestion, we determined the density of *E. anophelis* SCH814 cells in larval mosquitoes and incubation solution (sterile water). Compared to initial cell densities at time zero, 30%, 46%, 30%, and 43% of initial *E. anophelis* cells were detected in *A. stephensi* larval guts after being transferred into sterile water for 1 h, 1.5 h, 2 h, and 2.5 h, respectively (Fig. 2A). At these same sampling times and compared to time zero, 32%, 39%, 32%, and 37% of SCH814 were detected in the incubation solution. From these findings, overall, about 23% of SCH814 cells in *A. stephensi* were estimated to have been digested after 2.5 h of incubation in water (Fig. 2C). However, under the same conditions, a low level of residual SCH814 cells (ranging from 6.2% to 15.7% of the initial number of cells) were found in larval *A. gambiae* after being transferred within 2.5 h (Fig. 2A), while ca. 22% to 27% of ingested SCH814 cells were excreted into water (Fig. 2B). The digestion rate of SCH814 in *A. gambiae* larvae was estimated to be 71% after 2.5 h of incubation in sterile water (Fig. 2C). For *Aedes triseriatus*, the NanoLuc activity of SCH814 showed great variation (Fig. 2A) in larvae during the incubation time (ranging from 3% to 41%). However, only a small portion of the ingested *E. anophelis* cells (5% to 12% of replicates) were detected in water (i.e., excreted) during the incubation period (Fig. 2B). The digestion rate in *Aedes triseriatus* was estimated to be 85% after 2.5 h (Fig. 2C). Collectively, *E. anophelis* cells were more resistant to digestion in larval *A. stephensi* than in *A. gambiae* and *Aedes triseriatus* during the 2.5-h incubation period in water (Fig. 2C).

Association of *E. anophelis* with selected mosquitoes. Patterns of colonization of the host by *E. anophelis* were investigated in *A. gambiae*, *A. stephensi*, and *Aedes triseriatus* individuals by using GFP-labeled *E. anophelis* strain SCH837. After initial incubation of bacteria in second-instar larval mosquitoes at the concentration of 5×10^8 cells/ml for 24 h, the bacterial density in second-instar *Anopheles* was estimated at 5.7×10^5 cells/larva. Approximately 1.8×10^4 and 4.2×10^2 GFP-tagged *E. anophelis* cells were detected in *A. gambiae* fourth-instar larvae and pupae, respectively (Fig. 3). The adult mosquitoes retained up to 8×10^4 cells/mosquito, indicating some of the introduced *E. anophelis* survived digestion and propagated in *A. gambiae* (Fig. 3). Similar results were observed for *A. stephensi* (Fig. 3). For *Aedes triseriatus*, we only detected 3.0×10^4 and 1.1×10^2 GFP-tagged *E. anophelis* cells in larval mosquitoes at the second and fourth instar stages (Fig. 3). On average, less than 10 SCH837 cells could be detected in pupae and adult stages (Fig. 3).

When NanoLuc *E. anophelis* cells (SCH814) were introduced in the larval stage (second instar), they were readily detected in adult mosquitoes, with an 82% infection rate in *A. stephensi* (27/33 adults at 3 days postemergence) (Table 2). Our data further indicated there was no significant difference between female and male adult mosquitoes in retention of *E. anophelis* cells when feeding with 10% sucrose (see Fig. S4 in the supplemental material). The infection ranges of mosquitoes were next evaluated by introducing strain SCH814 into *A. gambiae* and *Aedes triseriatus* (second-instar larvae). The infection rate was ~71% (37/52) for *A. gambiae*. Only one was detected with introduced *E. anophelis* infection among the 30 adult *Aedes triseriatus* mosquitoes (Table 2). When SCH814 was introduced into mosquitoes in the adult stage (fed 10% sucrose), up to 96% (48/50) and 98% (54/55) of adult *A. stephensi* and *A. gambiae* mosquitoes were found to be carrying SCH814, respectively; for *Aedes triseriatus*, we detected luciferase activity in 88 of 99 randomly sampled mosquitoes.

Effects of sugars and amino acids on growth of *E. anophelis in vitro* and *in vivo*. Very little information is available about the nutrient requirements for flavobacteria in mosquitoes. Effects of physiological factors, including various nitrogen (amino acid) and carbon sources simulating the mosquito gut environment on flavobacterial growth, were investigated (Fig. 4 and 5). No significant *E. anophelis* growth was detected when cultured in M9 or SD (data not shown). To determine if *E. anophelis* needs specific amino acids for cell growth, 17 selected amino acids were individually tested in M9 medium (Fig. 4). Arginine (4 mM) was the only amino acid that significantly increased cell density (≥3-fold), in-

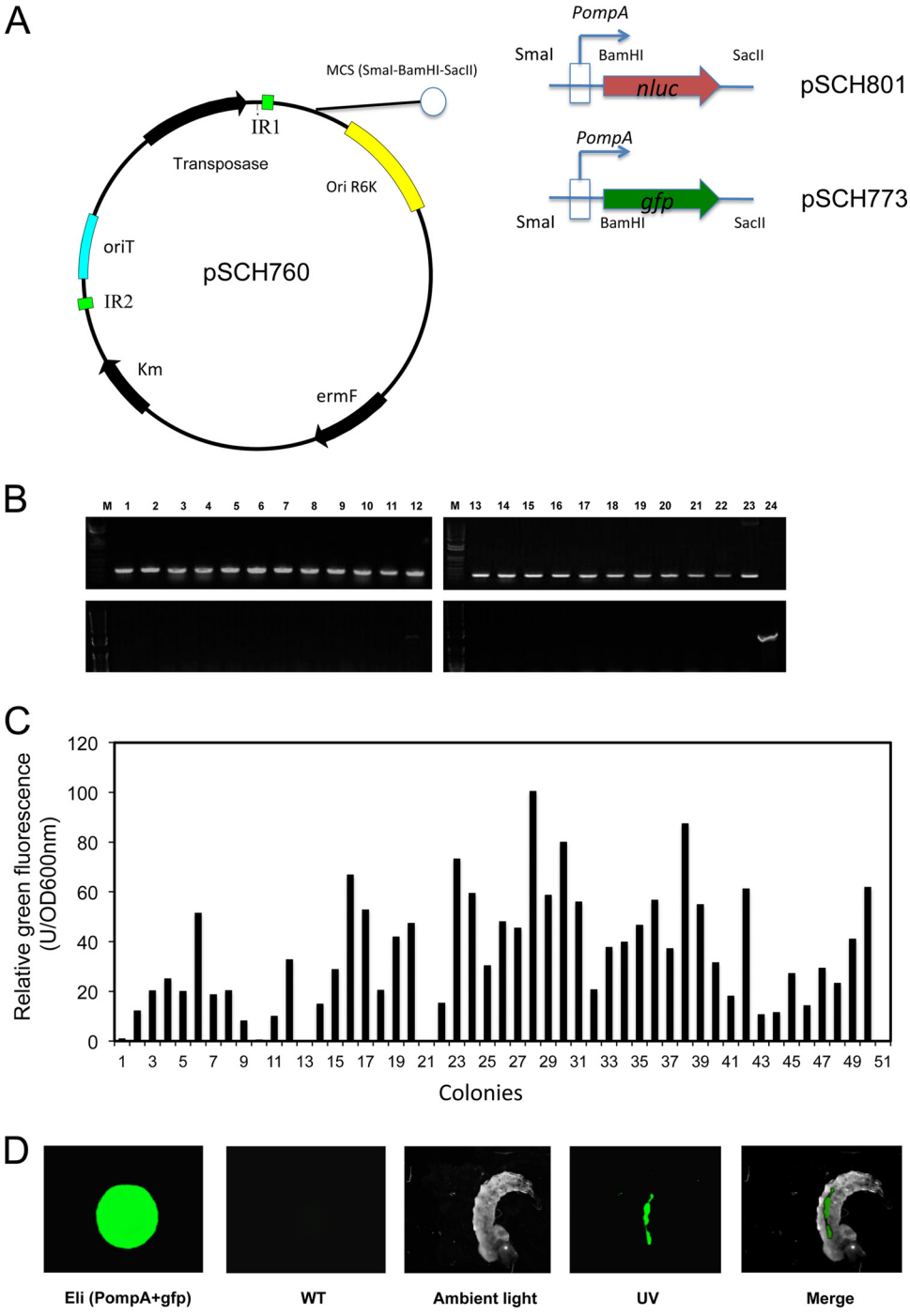

FIG 1 Reporter strain construction and demonstration of *E. anophelis* cells tagged with GFP and their ingestion by mosquito larvae. (A) Diagram of the pSCH760 construct. pHimarEm1(MCS) was modified with a multiple-cloning site (SmaI-BamHI-SacII); the expression cassettes P*ompA*+*gfp* or P*ompA*+*nluc* were inserted into SmaI and SacII sites on pSCH760 to generate pSCH773 and pSCH801, respectively. (B) The transposon incorporated into the *E. anophelis* MSU001 chromosome. (Upper panel) PCR screening results for the Em-resistant transconjugants, using the primers Walker140 and Walker141. Lane M, molecular marker; lanes 1 to 22, DNA fragments amplified from Em-resistant transconjugants; lane 23, positive control (pSCH760 as the amplification template); lane 24, negative control (*E. anophelis* MSU001). (Lower panel) Presence of transposase, determined using primers Walker186 and Walker187. Lanes 1 to 22, the same transconjugants as in the upper panel; lane 23, negative control (*E. anophelis* MSU001); lane 24, positive control (pSCH760 as the amplification template). (C) Quantitative analysis of *E. anophelis* emitting GFP fluorescence. Transconjugants were first screened under UV, and the fluorescent colonies were next quantified using fluorometry. The brightest colony was chosen for further study. (D) The cultures carrying the GFP reporter were incubated with *Anopheles* mosquito larvae for 2 h, and the larvae were observed by using epifluorescence microscopy. The control was *E. anophelis* MSU001 cells.

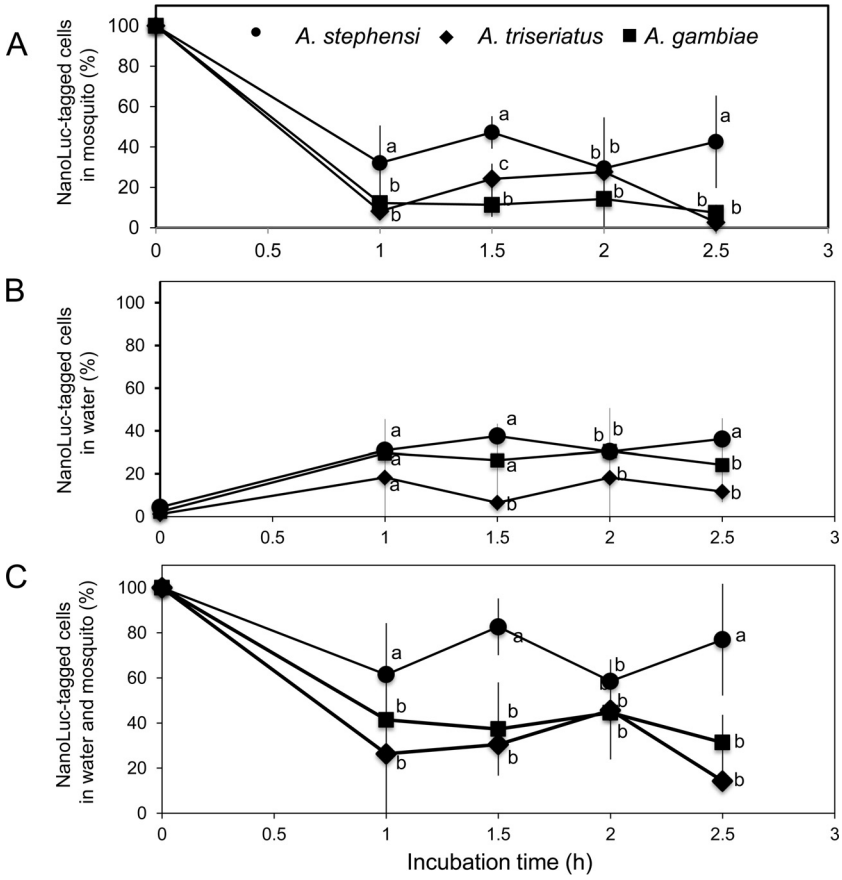

FIG 2 Digestibility analysis of *E. anophelis* by *Aedes triseriatus*, *A. gambiae*, and *A. stephensi* larvae. (A) Larval mosquitoes fed SCH814 cells were pooled (4 at each time point), homogenized, washed, and subjected to the NanoLuc activity assay. Cell densities at the different time points were normalized to the initial cell densities in corresponding mosquitoes at time zero. (B) Cells in the water were sampled, washed with PBS by centrifuging, resuspended in PBS, and subjected to the NanoLuc activity assay. Cell densities at the different time points were normalized to the initial cell densities at time zero. (C) The NanoLuc-tagged cells recovered from mosquitoes and water samples were quantified and normalized to those at time zero. Values are means ± standard deviations; triplicate experiments were performed. Significant differences among *Aedes triseriatus*, *A. gambiae*, and *A. stephensi* samples at each time point were determined by using PROC GLM. Different letters (a, b, and c) indicate significant differences in NanoLuc-tagged cell densities among these samples at each time point ($P < 0.05$). Means with the same letters indicate that no statistically significant difference was observed for these samples ($P > 0.05$).

dicating it is a critical amino acid for *E. anophelis*. *A. stephensi* mosquitoes were fed 10 mM arginine in 10% sucrose sugar meal to test if arginine affected *E. anophelis* growth *in vivo*. Compared to controls (fed 10% sucrose without arginine), NanoLuc-tagged *E. anophelis* cells significantly increased up to 100% ($P < 0.001$) in *A. stephensi* mosquitoes at 24 h when the sucrose was supplemented with 10 mM arginine (Fig. 4). However, there was no significant difference ($P > 0.05$) in density of NanoLuc-tagged bacteria between mosquitoes fed with arginine and fed sucrose without arginine after 72 h (Fig. 4).

Several sugars, including those possibly present in the normal diet of larval and/or adult mosquitoes (such as plant or animal detritus in water, sediments, or plant saps) were amended to investigate their effects on bacterial cell growth *in vitro* on M9 medium. The highest cell growth was observed in cultures supplemented with glucose, fructose, mannose, or glycerol, with glucose as the best carbon source. However, there was no significant difference for growth of NanoLuc-tagged *E. anophelis* (around 1.3×10^5 cells/mosquito in *A. stephensi*) *in vivo* between the mosquitoes fed with glucose (10%) and those fed with sucrose (10%) (Fig. 5).

Effects of animal blood on *E. anophelis* growth *in vivo* and *in vitro*. Different concentrations of horse blood (calculated packed cell volume [PCV], 0, 0.4%, 2.1%, 4.1%, 8.2%, and 12.3%) were tested for effects on *E. anophelis* SCH814 cell growth in modified M9 medium (Fig. 6A). Cell growth significantly increased with concentration of blood. Compared to controls, the number of cells doubled when the culture was supplemented with 8.2% PCV horse blood. The growth of *E. anophelis* SCH814 *in vivo* was also evaluated in mosquitoes fed a blood versus sugar meal. As shown in Fig. 6B, the number of introduced *E. anophelis* cells in blood-fed mosquitoes (3.4×10^5 cells/mosquito) was significantly higher than that in sugar-fed ones (5.7×10^4 cells/mosquito) after a 24-hour feeding ($P < 0.01$). However, at 4 days post-blood meal, *E. anophelis* cells decreased to a density of 8.5×10^4 cells/mosquito, similar to that in sugar-fed mosquitoes (Fig. 6B).

DISCUSSION

Mechanistic studies on bacterial colonization into the gut of mosquito hosts have emphasized microbial community analysis and adaptation of the bacteria to the gut environment (4, 23–25). The microbiota in mosquito guts has been revealed to be diverse and dynamic and greatly dependent on the host species, mosquito habitat, developmental stages, diet, and immune status (24). However, despite the diversity and dynamics of the assemblage of

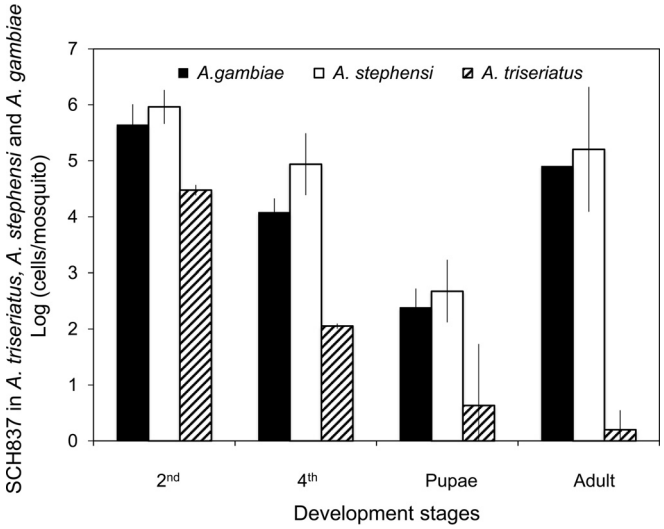

FIG 3 Association of introduced *E. anophelis* with mosquitoes. Cells tagged with GFP were introduced to *A. gambiae*, *A. stephensi*, and *Aedes triseriatus* second-instar mosquito larvae. The numbers of CFU were counted and calculated by plating homogenized mosquito samples (pools of 5 mosquitoes) on LB plates containing Em. Values are means ± standard deviations; triplicate experiments were performed.

the gut microbiome, a set of "core taxa" are present and mostly consist of *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (1, 25). Among them, bacteria of the phylum *Bacteroidetes* are ubiquitously and predominantly distributed in several vector mosquitoes, such as *A. gambiae* (up to 86%), *Aedes aegypti* (up to 40%), and *A. stephensi* (up to 33%) (7, 8). Yellow or orange colonies were the most dominant ones observed when we plated the mosquito midgut contents from lab-reared *A. stephensi* and *A. gambiae*, and these colonies provided isolates of culturable microbes for our studies. The 16S rRNA analysis showed that representative isolates were *Elizabethkingia* spp. and *Chryseobacterium* spp. We focused on one of the isolates, *E. anophelis* MSU001, because it grew well in LB medium and predominated for adult *A. gambiae* and *A. stephensi*. Molecular phylogenetic analysis of this primary isolate showed 99% identity with those from *E. anophelis* or *E. meningoseptica*, demonstrating that this strain isolated from the MSU insectary was similar to those isolated from mosquitoes in Europe and Africa (see Fig. S1 in the supplemental material). Due to their close phylogenetic associations with, as well as their dominance in, vector mosquitoes, *Elizabethkingia* and related bacteria have been proposed to play important physiological roles in mosquito biology and, further, might serve as potential control agents for intervening in malaria parasite development and subsequent transmission (10, 25). Generally, the invertebrate animal-associated microbiota have been demonstrated to profoundly affect their hosts in a wide variety of ways, such as metabolism and immunity (26, 27). Despite their ubiquity, our understanding of the physiological functions of *Elizabethkingia* species and related bacteria in vector mosquitoes is extremely limited. Further investigation of these commensals in mosquitoes could allow us to elucidate their physiological functions and explore their potential as paratransgenic tools for control of parasite transmission (7, 13). However, effective tools for molecular manipulation of these bacteria had heretofore been lacking, hampering investigations of meaningful interactions.

The superactive *mariner* transposon (pHimar) is widely used for mediating random mutagenesis of Gram-negative bacteria, such as *Haemophilus*, *Shewanella*, and *Delftia* species (28–30), and Gram-positive bacteria, such as *Listeria*, *Mycobacterium*, and *Bacillus* species (31–33). Once this transposon was equipped with the selective markers functional in *Bacteroidetes*, it was successfully utilized for mutagenesis analysis of various bacteria in the phylum *Bacteroidetes*, such as *Flavobacterium*, *Bacteroides*, *Riemerella*, and *Cytophaga* (34–37). In this study, we extended its utilization to the insertion of gene cassettes of interest into *E. anophelis* strains, including genes expressing the sensitive reporters GFP and NanoLuc. The expression cassettes P*ompA*+*gfp* and P*ompA*+*nluc* were assembled into the pHimarEm1 transposon and integrated into the *E. anophelis* chromosome successfully. Compared to the cell-tagging strategy that entails integration of reporters on plasmids, delivery of the gene by the transposon mechanism has several advantages. First, the chromosomal insertion of reporters avoids gene loss after propagation for generations in the host without selection pressure. Second, unlike other transposons with instability in host chromosomes, the transposase of pHimarEm1 (positioned outside the insertion sites) is not introduced into the chromosome, thus conferring considerable stability through immobilization (34). Third, when the P*ompA* expression system, a strong constitutive promoter in various flavobacteria, was used to promote expression of exogenous genes, it caused cell toxicity because of overexpression of the genes in multiple-copy plasmids (38). Single insertion of this expression system alleviated this problem. Fourth, the insertion sites of pSCH801 (NanoLuc) or pSCH773 (GFP) in SCH814 or SCH837, respectively, characterized by sequencing gene fragments on the recovered transposon, did not interrupt critical metabolism, such that cell growth was normal compared to the wild type. The GFP-tagged cells allowed us to qualitatively investigate the localization of the ingested bacteria *in situ* (Fig. 1) and quantitatively study bacterial colonization in mosquitoes using culture-based assays (Fig. 3). The NanoLuc reporter, functional in *F. johnsoniae* and *Flavobacterium hibernum*, has proven to be the most sensitive reporter so far in *Bacteroidetes* (38). Here, we have demonstrated that it is an excellent reporter in *E. anophelis*. NanoLuc reporter activity in *E. anophelis* varied negligibly in the different growth phases; detection for target cells based on the NanoLuc determination ranged from 5×10^2 to 5×10^8 cells/ml with good linearity, providing a reliable standard curve (see Fig. S3 in the supplemental material). The NanoLuc reporter activity representative of living *E. anophelis* was detected in mosquitoes at up to 14 days (see Fig. S4 in the supplemental material). These attributes are important when

TABLE 2 Infection rates of SCH814 in *A. gambiae*, *Aedes triseriatus*, and *A. stephensi* mosquitoes

Introduced stage[a]	Infection rate (no. infected/total)		
	A. gambiae	*Aedes triseriatus*	*A. stephensi*
Larvae	71% (37/52)	3% (1/30)	82% (27/33)
Adult	98% (54/55)	89% (88/99)	96% (48/50)

[a] To introduce *E. anophelis* for the larvae infection study, NanoLuc reporter bacteria were fed to second-instar larval mosquitoes (see Materials and Methods). Pupae were transferred into sterile water for adult emergence. Once adult mosquitoes emerged, they were randomly sampled and subjected to the NanoLuc reporter assay. For the adult infection study, mosquitoes were fed a suspension of *E. anophelis* in 10% sucrose overnight. The adults were subjected to the NanoLuc reporter assay after 3 days.

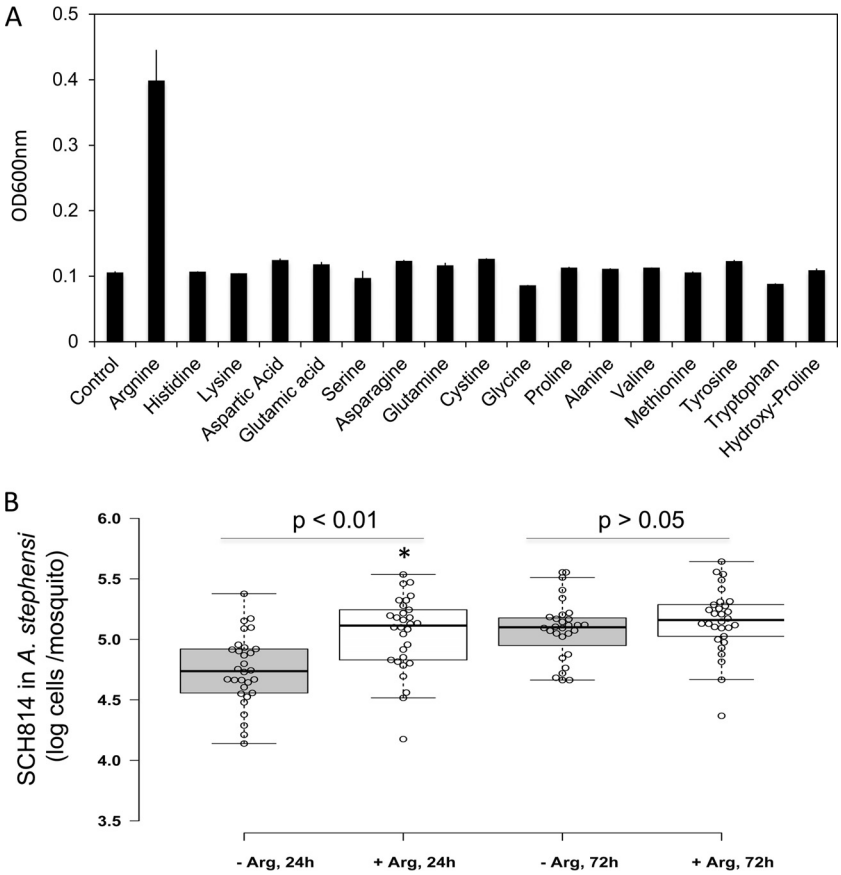

FIG 4 Effects of amino acids on SCH814 growth *in vivo* and *in vitro*. (A) Selected amino acids at 4 mM (final concentration) were individually added to M9 medium with glucose. After 24 h of incubation at 30°C, the cells were subjected to optical density determinations at 600 nm. (B) *A. stephensi* mosquitoes were fed 10% sucrose supplemented with SCH814 for 24 h (NanoLuc reporter strain). After the adult mosquitoes emerged, they were fed 10% sucrose with 10 mM arginine or 10% sucrose without arginine. After 24 h and 72 h, 30 mosquitoes were randomly sampled from sucrose without arginine treatment at each time point. Under the same conditions, 28 and 30 mosquitoes were sampled from sucrose with 10 mM arginine treatment, respectively. Mosquitoes were homogenized and subjected to NanoLuc assays. Significant differences between arginine addition and no-arginine addition samples were determined by using PROC GLM; significantly different cell densities are denoted by an asterisk ($P < 0.05$).

research requires that reporters in living organisms be sensitive, stable in expression, and not unduly affected by environmental factors (39, 40).

Even though *E. anophelis* has been described as either an endosymbiont or commensal in *A. gambiae*, the infection range and fitness of *E. anophelis* in this species or, comparatively, in other mosquito species, remained unclear (5, 8). Multiple pathways (vertical, horizontal, and transstadial) have been demonstrated for *Elizabethkingia* species transmission in mosquitoes (5, 8, 41). For example, *E. meningoseptica* bacterial cells were detected in *A. gambiae* ovarian tissues and embryos, and they successfully colonized the F_1 generation, indicating vertical transmission (5). These experiments involved axenic mosquitoes (i.e., those treated by antibiotics) and did not consider the difference in competency between introduced bacteria and indigenous ones. Our studies here demonstrated that *E. anophelis* efficiently spread to *A. gambiae* and *A. stephensi* populations with high rates of infection (71% and 82%, respectively) when introduced to the larval stages by the feeding route. Introduction of *E. anophelis* cells in both larval and adult stages was feasible for infection of *A. stephensi* because the infection rate in adult *A. stephensi* mosquitoes (96%) was comparable to that in the larval stages (82%). Further, colonization and perpetuation of *E. anophelis* in guts of conventionally reared mosquitoes without either prior antibiotic treatment or concurrent antibiotic selection pressure suggest that it is a promising bacterial species for paratransgenesis applications.

The dynamics of *E. anophelis* infection and survival in *Aedes triseriatus* were quite different from those in the two *Anopheles* species (Fig. 2 and 3). Infection reached a high rate when SCH814 cells were provided to the adult stages in sugar or blood meals, but the infection rate in *Aedes triseriatus* was substantially lower than those in *A. gambiae* and *A. stephensi* when the bacteria were fed to larvae, indicating that *E. anophelis* varies in survival by stage across these three mosquito species (Table 2 and Fig. 3). Flavobacteria are well known to spread ubiquitously in nature and widely reside in mosquito habitats (42). Among the few groups of bacteria common in *Aedes* and *Anopheles* guts of their various development stages, some flavobacteria (such as *E. anophelis*) seem to have evolved mechanisms to adapt to the harsh midgut environment of mosquitoes, despite a certain degree of digestibility (8). However, surveys of the microbial community in *Culex* species mosquito guts showed flavobacteria were not associated with them (43). The same observation was also reported for the mosquito *Georgecraigius atropalpus*, even though the species was reared under similar

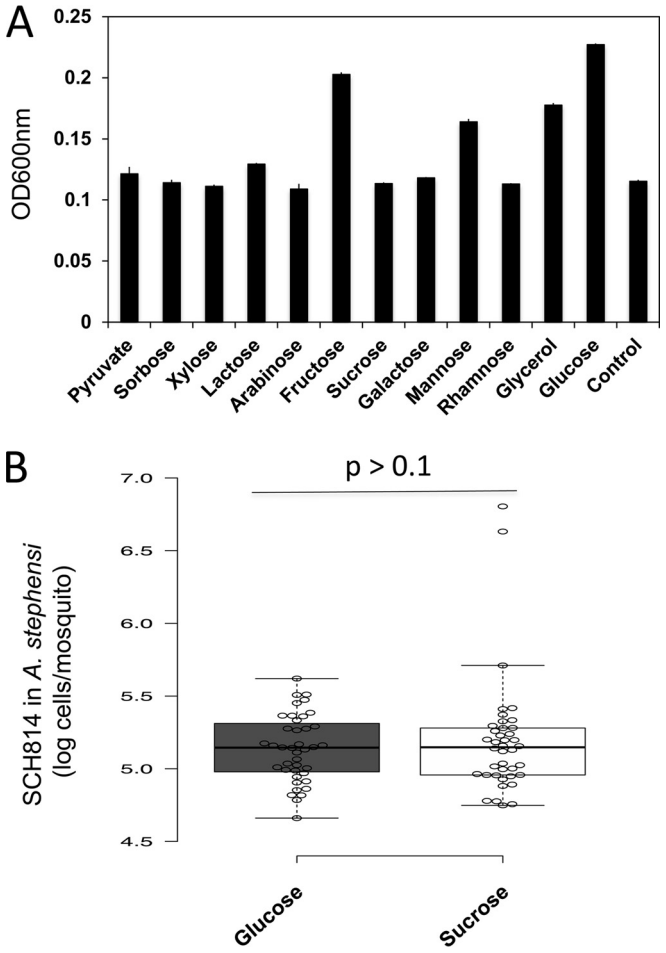

FIG 5 Effects of carbon source on SCH814 growth *in vivo* and *in vitro*. (A) Selected carbon sources at a 0.5% (wt/vol) final concentration were added to M9 medium. After 24 h of incubation at 30°C, the cells were subjected to optical density determinations at 600 nm. (B) Second-instar larvae (*A. stephensi*) were inoculated with SCH814 (NanoLuc reporter strain). After the adult mosquitoes emerged, they were fed 10% glucose or 10% sucrose. Thirty mosquitoes from each treatment group were randomly sampled, homogenized, and subjected to NanoLuc assays. Significant differences between glucose and sucrose samples were determined by using PROC GLM.

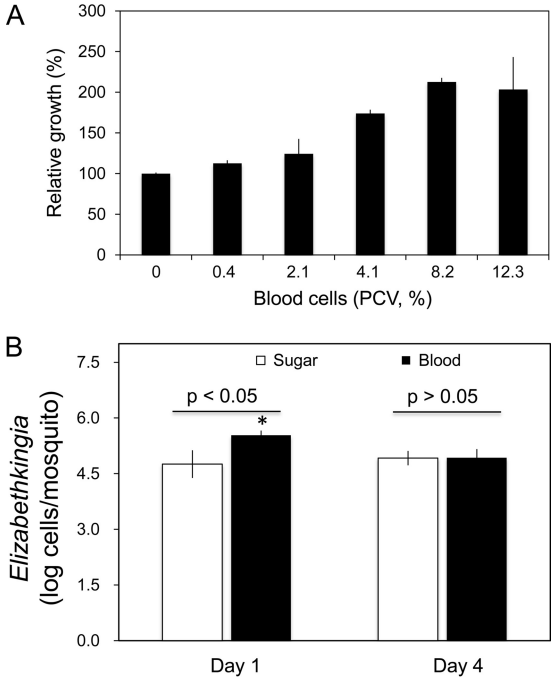

FIG 6 Effects of animal blood on SCH814 growth *in vivo* and *in vitro*. (A) Effect of different concentrations of animal blood cells on SCH814 growth in M9 medium *in vitro*. SCH814 cells were cultured in M9 medium (with glucose) supplemented with various concentrations of horse blood cells. After incubation at 30°C for 24 h, SCH814 cells were estimated by the determination of NanoLuc activity; the relative growth was expressed as the percentage relative to the control (without supplementation with horse blood cells, set as 100%). Values are means of single measurements from triplicate experiments (± standard deviations). (B) Effect of sugar and blood meals on SCH814 growth in mosquitoes. A suspension of SCH814 cells in 10% sucrose was fed to *A. stephensi* mosquitoes for 24 h in order to introduce NanoLuc-tagged bacteria. The mosquitoes were then given sugar meal (10% sucrose) or blood meal via a membrane apparatus (see Materials and Methods). Four mosquitoes from each treatment group were sampled for assay of NanoLuc activity on day 1. Under the same testing conditions, 8 mosquitoes from each treatment group were sampled on day 4. Significant differences between sugar and blood meals were determined by using PROC GLM and are denoted by an asterisk ($P < 0.05$).

conditions as those for *A. gambiae* and *Aedes aegypti* (8). Microbial community structure in the mosquito gut is regulated by many complicated factors, such as mosquito species, developmental stages, immune status, and diet (27). However, very few studies have investigated whether the dominant members of the gut microbiota could be digested by larval mosquitoes. *Flavobacterium hibernum* cells were quickly and thoroughly digested by larval *Aedes triseriatus*, showing that they were a food source for mosquitoes, rather than a gut commensal or symbiont (38). Some *E. anophelis* cells were digested in larval mosquitoes in our experiments, and indeed digestion varied among the three species, with a high rate in *Aedes triseriatus* and *A. gambiae*. Different digestibilities may explain why *E. anophelis* has differential persistence in various mosquito species, which raises questions regarding survival and proliferation of different flavobacteria in the mosquito midgut environment (8, 25). However, as demonstrated in Fig. 3, a residual portion (i.e., undigested and GFP-tagged *E. anophelis*) passed via transstadial transmission from larvae through pupae to the adults in *A. stephensi* and *A. gambiae*, indicating that *E. anophelis* can adapt to the *A. stephensi* and *A. gambiae* midgut environments and survive the several molting events during larval stages, as well as hydrolytic processes during metamorphosis, during which most bacteria are typically eliminated (8). The bacterial digestibility by mosquitoes should be one of the important factors affecting their durability in the insect midgut (44). However, further experiments should be conducted to examine how different mosquitoes selectively preserve their symbionts and transmit them to future generations (25).

The dominant flavobacteria in the mosquito gut may have a beneficial role for insects, e.g., *Chryseobacterium* rescued axenic larval mosquito development (8). Like a few other Gram-negative bacteria, *Chryseobacterium* species have been suggested to provide unknown signaling molecules that are critical for regulating larval growth processes (8). In addition, such bacteria could possibly supply necessary nutrients that are important to larvae, such as a nitrogen source, or vitamins or other unknown factors. On the other hand, for commensal *E. anophelis* cells to live in the adult

mosquito, it might also interact with host and other midgut microbes to maintain metabolic activities. *Elizabethkingia* have been assumed to have a good ability to use the sugars obtained by mosquitoes, compared to the less-dominant bacteria (2). However, *E. anophelis* did not utilize sucrose as a sole carbon in M9, but it propagated in the mosquitoes when fed sucrose (Fig. 5) (1). Sucrose is one of the most common sugars ingested by mosquitoes from plant sap or floral nectar and is widely used for rearing mosquitoes in the lab (45). It is unclear why *E. anophelis* did not grow well when sucrose was used as the sole carbon source, though there are some genes encoding α-glucosidases which can hydrolyze sucrose to release α-glucose (46). However, the mosquito hosts may directly participate in this process, because they secrete several α-glucosidases into the midgut (47). *E. anophelis* may lack transporters for sucrose.

E. anophelis did not utilize most of the selected amino acids to support cell growth (Fig. 4). Arginine was identified as a critical amino acid for *E. anophelis* for metabolism when cultured in defined medium. Supplementation of arginine in the sugar diet significantly increased *E. anophelis* growth in *Anopheles* mosquito midgut after 24 h, indicating that arginine is also an important amino acid for supporting *E. anophelis* cell growth *in vivo*. Even though we did not generate experimental evidence that indicated the specific nitrogen source for *E. anophelis* growth in the mosquito midgut, we assume amino acids may partially come from other microbes or from the mosquito host. *E. anophelis* could lyse a wide range of Gram-positive and Gram-negative bacteria, which may allow *E. anophelis* to more efficiently recycle necessary nutrients, such as amino acids (41). It has been reported that bacterial symbionts in insects obtain necessary nutrients (nitrogen or carbon sources) from hosts or other microbes (48). For example, bacilli predominant in the honey bee gut need to take up amino acids from diet or from biosynthesis by microflora (49). Lee et al. proposed that *Gammaproteobacteria* and *Actinobacteria* provide all essential and other nonessential amino acids for bacilli (49). *E. anophelis* could also obtain necessary nutrients (such as amino acids) from lysed animal erythrocytes in mosquito midguts (Fig. 6). The finding of a 6.0-fold-higher level of *E. anophelis* cells after the blood meal agrees with previous observations that flavobacteria dramatically increase proportionately after blood ingestion (1). Kukutla et al. demonstrated that *E. anophelis* has hemolytic activity *in vitro* (2). Many genes encoding putative hemolysins and heme-degrading proteins are present in the *E. anophelis* genome. Data presented here and by others support the idea that *E. anophelis* is involved in digestion of erythrocytes, which could influence mosquito fecundity. It should be noted that *E. anophelis* cell growth was not significantly inhibited when erythrocytes were added to M9 medium, indicating that this bacterium has evolved mechanisms to tolerate high oxidative pressures related to blood meal utilization (2).

We successfully developed the techniques for integrating foreign DNA into the chromosome and expressing genes of interest in commensal *Elizabethkingia*. This development will provide alternative avenues to develop novel biocontrol agents for mosquito-borne diseases. The reporter strains developed in this study will allow us to understand bacterial infection, fitness, and fates in various vector mosquitoes. The NanoLuc-based or GFP-based reporter construct will also facilitate studies of gene regulation and *in vivo* cell localization. In summary, the availability of the culture conditions, sensitive reporters, and transposons described in this study will deepen our understanding of the interactions between mosquitoes and bacteria or between bacterial species under complex conditions. Future studies should focus on identifying effective effector molecules to use in the expression system and developing methods to increase the stability of transgene expression over time.

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