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Influence of *Salmonella enterica* Serovar Typhimurium *ssrB* on Colonization of Eastern Oysters (*Crassostrea virginica*) as Revealed by a Promoter Probe Screen

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Although *Salmonella* has been isolated from 7.4 to 8.6% of domestic raw oysters, representing a significant risk for food-borne illness, little is known about the factors that influence their initial colonization by *Salmonella*. This study tested the hypothesis that specific regulatory changes enable a portion of the invading *Salmonella* population to colonize oysters. An *in vivo* promoter probe library screen identified 19 unique regions as regulated during colonization. The mutants in the nearest corresponding downstream genes were tested for colonization defects in oysters. Only one mutation, in *ssrB*, resulted in a significantly reduced ability to colonize oysters compared to that of wild-type *Salmonella*. Because *ssrB* regulates *Salmonella* pathogenicity island 2 (SPI-2)-dependent infections in vertebrate macrophages, the possibility that *ssrB* mediated colonization of oyster hemocytes in a similar manner was examined. However, no difference in hemocyte colonization was observed. The complementary hypothesis that signal exchange between *Salmonella* and the oyster's native microbial community aids colonization was also tested. Signals that triggered responses in quorum sensing (QS) reporters were shown to be produced by oyster-associated bacteria and present in oyster tissue. However, no evidence for signal exchange was observed *in vivo*. The *sdiA* reporter responded to salinity, suggesting that SdiA may also have a role in environmental sensing. Overall, this study suggests the initial colonization of live oysters by *Salmonella* is controlled by a limited number of regulators, including *ssrB*.

Market surveys in the United States have isolated *Salmonella* in 7.4 to 8.6% of raw, market-ready oysters, with local contamination rates as high as 77% within a single sample (1–3). Because oysters are typically consumed raw or lightly cooked, they present a significant risk for exposure to live pathogens (4, 5). Oysters are capable of sustaining high filtration rates and are known to concentrate bacteria, including pathogens, present in the environment (6–8). Colonization is rapid; detectable contamination occurs in as little as 15 min following exposure to water containing *Salmonella* (9). Despite the clear evidence that *Salmonella* colonizes oysters, the mechanisms mediating *Salmonella* colonization of, and persistence inside, shellfish remain unclear.

Oysters are typically colonized by a diverse microbial community, with 90% of the inhabiting bacteria being associated with the digestive tract. The composition of oyster microbiota is derived, but clearly differs, from the surrounding seawater and becomes progressively dissimilar from the seawater from the stomach to the lower intestine (10). This divergence in community structure indicates selection for certain species and suggests that the ability to resist shedding is responsible for the enhanced persistence. Experimental contamination of oysters by *Salmonella* typically results in a rapid initial colonization at a density 1- to 2-log-fold below that of the inoculum, followed by a decrease within the first 24 to 48 h and then persistence by a remnant population, which stays stable or only slowly declines over time (6, 11–15). However, *Salmonella* is more persistent than other enteric bacteria, such as *Escherichia coli*, and can survive within oysters at detectable levels for at least 60 days (6, 11, 12). This ability could allow *Salmonella* contamination to persist beyond harvest closures and could lead to postharvest exposure following reopening of shellfish harvesting areas (15).

The factors that allow certain bacterial species to become established on and persist in oysters while others are shed or destroyed are generally unknown, as few studies of the genetic determinants of bacterial colonization of oysters have been reported. Targeted studies have identified possible roles for surface attachment via pili, cell surface proteins such as OmpU, and virulence factors like metalloproteases in the persistence of *Vibrio* spp. on oysters (16–19). The only known study of the persistence of *Salmonella* in live oysters investigated effectors within *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 but found no effect (20).

Interspecies and even interkingdom signaling systems are used by bacteria to alter their gene expression in response to the recognition of specific signal molecules. The production of and interference with signal molecules are both common strategies employed during surface colonization in the marine environment (21). "Eavesdropping" on the signals of established microbial communities is another way for invading bacteria to gain an advantage when colonizing a new host (22). It remains unknown if

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cell-to-cell signaling occurs within the oyster microbiome; however, it seems likely, as many genera associated with oysters are known to produce *N*-acyl homoserine lactones (AHLs) and autoinducer 2 (AI-2) quorum-sensing signals. *Salmonella* is able to sense, but not produce, AHLs via the receptor encoded by *sdiA* and possesses a fully functional AI-2 signaling system encoded by the synthase *luxS* and receptor *lsrR* adjacent to the *lsrACDBFG* operon (23–25).

The primary goal of this study was to identify factors that allow *Salmonella* to become established within oysters and potentially contribute to seafood-related food-borne illness. We hypothesized that specific genetic elements would allow a portion of the *Salmonella* population to be established within oysters, by either resisting shedding or avoiding oyster defenses, which are responsible for the reduction of colonization density within the first 24 h following contamination (6, 11, 13). Because so little is currently known about how *Salmonella* interacts with marine invertebrates, the first approach was to screen a promoter probe library to identify possible promoters that are differentially regulated during the initial colonization of live oysters. The second approach examined the role of the GacS/GacA two-component system as well as quorum sensing via the AHL, AI-2, and putative QseB/C signaling systems during colonization. The *in vivo* expression of the selected promoters was determined using recombinase-based *in vivo* expression technology (RIVET) reporters. Defined mutants of these systems were used to determine a competitive phenotype compared to the wild-type strain.

MATERIALS AND METHODS

Bacterial strains and culture. *Salmonella* strains were grown at 37°C in Luria broth (LB) with antibiotics as necessary. Antibiotics were used at the following concentrations; ampicillin, 200 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 10 μg/ml. All strains and plasmid used in this study are listed in Table 1. All primers used are listed in Table S1 in the supplemental material. Oyster agar (OA) was prepared using a modified protocol (1, 2). Briefly, aseptically shucked oyster meats were blended in 2 volumes of sterile 1/2-strength artificial seawater (ASW) and extracted by boiling for 30 min. The resulting broth was filtered through mesh screens and 11-μm cellulose filter paper under vacuum (Whatman no. 1; GE) to remove coagulated proteins and debris. The filtrate was brought up to the original volume with deionized water, 1.5% agar (wt/vol) was added, and the medium was autoclaved. ASW (1/2 strength) was prepared from commercial aquarium salts in distilled water at a concentration of 16 ppt (either 15.23 g/liter of Instant Ocean [Aquarium Systems, Inc., Mentor, OH] or 17.47 g/liter of Coral Pro Salt [Red Sea, Eilat, Israel] salt mix was used depending on availability from local suppliers). Recovered samples were plated on xylose lysine deoxycholate agar (XLD) with antibiotics as necessary for the identification of *Salmonella*.

Oyster maintenance. Eastern oysters (*Crassostrea virginica*) were obtained from commercial sources in Apalachicola Bay or Cedar Key, FL, and transported to Gainesville, FL, in coolers with ice. Upon receipt, oyster shells were scrubbed under running tap water to remove mud and debris and acclimated in 1/2-strength ASW (16 ppt) at 22°C. Acclimation tanks were filtered (Whisper 10i; Tetra) and aerated to maintain water quality. Prior to use in assays, oysters were removed from the acclimation tank and rinsed under distilled water. Assays were performed in polystyrene bins with 5 liters of 1/2-strength ASW unless noted. All oyster infections were incubated at 22°C in 5-liter bins without filtration for 24 h before harvest. Oysters were considered active when either the shells were observed to open and close or feces were excreted into the bin.

***gfp*-labeled promoter probe library screen.** The previously constructed *Salmonella* promoter probe library consisted of random small fragments of the *S. enterica* 14028 genome cloned upstream of the promoterless *gfp* obtained by removing the Tn*5* promoter from the commercial pTurboGFP-B reporter plasmid (26). A negative control, pGFP-OFF, was created by self-ligating the promoterless pTurboGFP-B. A plasmid isolated from a colony with strong constitutive expression of green fluorescent protein (GFP) was selected as a positive control (pGFP-ON) and was later identified by sequencing as carrying a *dppA* promoter upstream of the promoterless GFP gene (27). Prior to the screening in live oysters, a 1-ml aliquot of the promoter probe library was amplified in 10 ml of LB with ampicillin at 37°C overnight. A 1-ml aliquot was analyzed via fluorescence activated cell sorting (FACS) using a FACSAria flow cytometer (Becton Dickinson, Franklin Lakes, NJ) in the University of Florida ICBR core lab to select for inactive promoters. Sorting gates for FACS were established using the positive and negative controls. 1,870,000 LB "off" cells were recovered, concentrated and grown overnight in 5 ml of LB at 37°C. A 500 μl sample of the population was analyzed by FACS to confirm promoter inactivity, and aliquots were saved as glycerol stocks for future screens.

The LB "off" library was subjected to three subsequent screens in live oysters. A 1-ml aliquot of the LB "off" library was amplified via overnight growth in 5 ml LB, washed and concentrated to 1 ml in 1/2-strength ASW. Two oysters in 10 liters of 1/2-strength ASW were exposed to the library via natural filtration. The library was recovered following 24 h incubation at 22°C by homogenizing the oysters in a blender with 50 ml of phosphate-buffered saline (PBS) for 60 s. The resulting homogenate was placed on ice and sequentially filtered through mesh screens and 11-μm cellulose filter paper (Whatman no. 1; GE) under vacuum to prepare for FACS. Active reporters were recovered, washed in LB to remove the capture buffer, and stored as a glycerol stock between infections. After the final selection, the recovered cells were washed and concentrated in 250 μl of LB. Serial dilutions (1/10) were prepared, and 10-μl aliquots were plated on LB ampicillin agar. Opaque (*gfp* off) colonies were saved for further analysis by transfer to 96-well plates (see Fig. S1 and S2 in the supplemental material).

The recovered colonies were subjected to further selection for oyster-specific activity by analyzing for the differential fluorescence activity of each strain following growth in LB or oyster homogenate for 24 h at 37°C. Ninety-six-well plates were inoculated from the master stock, and their fluorescence was analyzed using a multimode microtiter plate reader (Victor^3; PerkinElmer, Fremont, CA), equipped with Wallac1420 manager workstation software. To select the promoters most strongly associated with oysters, colonies with *gfp* activity that was 1 standard deviation above or below the in-plate mean when they were grown in oyster homogenate compared to LB were selected for further study. The reporter plasmids were recovered from the selected colonies of interest using a QIAprep Spin miniprep kit (Qiagen) and the putative promoters were identified from the plasmid by sequencing and alignment to the *S. enterica* serovar Typhimurium LT2 genome using BLAST.

Construction of *Salmonella* mutants. *Salmonella* deletion mutants were constructed via Datsenko and Wanner mutagenesis by removing the entire open reading frame (ORF) between the start and stop codon and replacing it with the *frt-kan-frt* cassette, which was amplified from plasmid pKD4 (28). The mutations were confirmed via PCR and transduced into *Salmonella enterica* serovar Typhimurium ATCC 14028 via phage P22 generalized transduction. To check for possible growth defects that could affect the coinfections, growth of each mutant was tested at either 22°C or 37°C for 12 or 8 h, respectively. Each hour, a 1-ml aliquot was removed from the culture, and the optical density at 600 nm (OD_{600}) was recorded using a spectrophotometer (Biospec Mini; Shimadzu).

RIVET reporters were constructed using the method of either Osorio et al. (29) (pGOA1193) or Merighi et al. (30) (λ*red*), as adapted for *Salmonella*. For pGOA1193 constructs, proper orientation of *tnpR* was confirmed via sequencing of the final construct using primer MT59. The orientation of λ*red* constructs was confirmed via PCR with the primer K2 and an internal primer specific to each construct (28). RIVET reporters constructed via the pGOA1193 method are ampicillin-resistant merodip-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
DH5α	*E. coli* ϕ80 *lacZ*ΔM15 *deoR endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi-1* Δ(*lacZYA-argF*)*U169*	Life Technologies
DH5α λ*pir*	DH5α λ*pir* phage lysogen	60
BW20767	*E. coli* K-12 RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant *leu*-63::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(ΔMluI)::*pir*$^+$ *thi*	61
14028	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
JS246	14028 *yje*P8103::*res1-tetAR-res1*	30
MJW129	*ssrB*::Cm^r	62
MHM51	ΔSPI-2 (Δ*ssrB-ssaU*::Kan^r)	M. H. de Moraes, submitted for publication
MM_011_C12	14028 Δ*phsA*::FRT-Kan^r-FRT	63
MM_009_E04	14028 ΔSTM0306::FRT-Kan^r-FRT	63
MM_019_C10	14028 Δ*luxS*::FRT-Kan^r-FRT	63
MM_073_D12	14028 Δ*lsrACDBF*::FRT-Kan^r-FRT	63
MM_015-G07	14028 Δ*lsrG*::FRT-Kan^r-FRT	63
BA612	14028 *sdiA*::Tn*3*	23
BA736	14028 *sirA*::Kan^r	64
JNS3206	JS246 *srgE-tnpR-lacZY*-Kan^r	65
JNS3216	JS246 *sdiA-tnpR-lacZY*-Kan^r	27
JNS3226	JS246 *srgE-tnpR-lacZY*-Kan^r *sdiA*::Tn*3*	65
JNS3236	JS246 *csrB-tnpR-lacZY*-Kan^r	B. Ahmer, unpublished data
CEC0015	JS246 *lsrG*::*tnpR-lacZY* Amp^r	43
CEC0018	JS246 *lsrG*::*tnpR-lacZY* Δ*luxS*::FRT-Kan^r-FRT Amp^r	43
CEC0026	JS246 P_{luxS}-*tnpR-lacZY* Δ*luxS*::FRT-Kan^r-FRT	43
CEC0035	14028 Δ*lsrACDBF* Δ*luxS*::FRT-Kan^r-FRT	43
CEC0001	JS246 *ssrB*::*tnpR-lacZY* Amp^r	This study
CEC0002	JS246 *csiE*::*tnpR-lacZY* Amp^r	This study
CEC0003	JS246 *yggB-tnpR-lacZY* Kan^r	This study
CEC0004	JS 246 STM4596::*tnpR-lacZY* Amp^r	This study
CEC0005	JS246 *pckA*::*tnpR-lacZY* Amp^r	This study
CEC0006	JS246 *yhdG-tnpR-lacZY* Kan^r	This study
CEC0007	JS246 STM0306::*tnpR-lacZY* Amp^r	This study
CEC0008	JS246 *glk*::*tnpR-lacZY* Amp^r	This study
CEC0009	JS246 *qor*::*tnpR-lacZY* Kan^r	This study
CEC0010	JS246 *yjfO*::*tnpR-lacZY* Amp^r	This study
CEC0011	JS246 STM11470::*tnpR-lacZY* Amp^r	This study
CEC0012	JS246 *pabC*::*tnpR-lacZY* Amp^r	This study
CEC0013	JS246 *dkgA*::*tnpR-lacZY* Amp^r	This study
CEC0014	JS246 *rfaZ-tnpR-lacZY* Kan^r	This study
CEC0019	JS246 *yibL-tnpR-lacZY* Kan^r	This study
CEC0020	JS246 *udp-tnpR-lacZY* Kan^r	This study
CEC0021	JS246 *dppA-tnpR-lacZY* Kan^r	This study
CEC0022	JS246 *phsA-tnpR-lacZY* Kan^r	This study
CEC0023	14028 Δ*yihX*::FRT-Kan^r-FRT	This study
CEC0024	JS246 *qseC-tnpR-lacZY*	This study
CEC0025	JS246 *qseC-tnpR-lacZY* Δ*luxS*	This study
CEC0027	14028 Δ*pabC*::FRT-Kan^r-FRT	This study
CEC0028	14028 Δ*yibL*::FRT-Kan^r-FRT	This study
CEC0029	14028 Δ*udp*::FRT-Kan^r-FRT	This study
CEC0030	14028 Δ*qseC*::FRT-Kan^r-FRT	This study
CEC0031	14028 Δ*yggB*::FRT-Kan^r-FRT	This study
CEC0033	14028 Δ*yhdG*::FRT-Kan^r-FRT	This study
Plasmids		
pTurboGFP-B	P_{lacZ}-TurboGFP (Amp^r)	Evrogen
p*dppA-gfp*	GFP "on" control	27
pCR2.1-TOPO	General cloning vector *lacZ*α (Kan^r Amp^r)	Invitrogen
pGOA1193	pIVET5n with promoterless *tnpR-lacZY*α (Amp^r)	29
pKD4	*oriR6K bla rgnB* FRT-Kan^r-FRT (Kan^r)	28
pKD46	*repA101*(Ts) *oriR101 araC* P_{araB} λ*red*(γ-β-*exo*)-*tL3* (Amp^r)	28
pCP20	*repA101*(Ts) λ_{pR}-Flp *ci857 (Amp^r Kan^r)*	66
pCE70	*oriR6K* FRT-promoterless *tnpR-lacZY*α (Kan^r)	30
pCE71	*oriR6K* FRT-promoterless *tnpR-lacZY*α (Kan^r)	30

loids. The RIVET reporters constructed by the λ*red* method are kanamycin resistant and consist of *tnpR* placed just downstream of the stop codon of the intact gene. The *qseBC* RIVET reporter, CEC0024, was constructed using the λ*red* method, placing *tnpR* immediately after the stop codon of *qseC* to avoid interrupting the operon. Strain CEC0025 was constructed by transducing the *qseC-tnpR*-Kan^r cassette from CEC0024 into the same JS46 Δ*luxS*::FRT (Flp recombination target) intermediate used to construct CEC0026. Genotypes of strains used and constructed are listed in Table 1.

Promoter regulation in live oysters measured via RIVET assays. Recombinase-based *in vivo* expression technology (RIVET) reporters utilize a heritable antibiotic-resistance phenotype which can "record" low levels of gene expression or signals which may occur only transiently during host colonization and have been shown to provide sensitive quantification of gene expression during *Salmonella* colonization of host environments (31). Results of RIVET assays are plotted as percent resolution. "Resolution" refers to expression of *tnpR* within the constructed RIVET reporter strains. In the RIVET reporters, *tetR* is flanked by two *res* sites. Once expressed, TnpR catalyzes recombination between the *res* sites, thus excising the tetracycline resistance marker. The loss of tetracycline resistance creates a permanent marker that is also passed to daughter cells. Because the resulting tetracycline-sensitive phenotype is inheritable, subsequent analysis of recovered cells provides a record of the portion of the population in which the target gene was expression *in vivo*.

Single oysters were inoculated with 1 ml of washed overnight culture. The *Salmonella* reporters were recovered by blending the shucked oyster as before and then plated on XLD with ampicillin or kanamycin at 37°C to select for RIVET reporters. Fifty colonies were patched from the recovery plate onto LB tetracycline at 37°C to determine the ratio of resolved colonies. As a control for the specificity of resolution to oyster colonization, 10 μl of the RIVET inoculum was also spotted onto LB agar, oyster agar, and 1/2-strength artificial seawater 0.3% (soft) agar (1/2 ASW) and incubated at 22°C for 24 h. Samples were streaked onto XLD with a sterile wire loop and analyzed in the same manner as the oyster samples.

Competitive fitness/coinfection assays in live oysters. The *in vivo* competitive fitness of each strain versus the wild type was determined by calculating a competitive index as described previously (32). Briefly, three oysters per 5-liter bin were inoculated with a roughly 50:50 mix of mutant to wild type prepared from 1/100-diluted overnight cultures. After 24 h, individual oysters were harvested by stomaching in Whirl-Pak bags (Nasco, Fort Atkinson, WI) with 50 ml of PBS in a Stomacher 4000 circulator (Seward, West Sussex, United Kingdom) at 260 rpm for 1 min. The resulting homogenate was plated on XLD at 42°C to limit growth of oyster commensals. A 1,000-fold dilution of the original inoculum was plated on XLD to determine the initial mutant-to-wild type ratio. Fifty individual colonies were patched from XLD to LB kanamycin, and the number of mutants was determined by counting the number of kanamycin-resistant colonies.

Changes in the mutant-to-wild type ratio between the inoculum and recovered samples were used to calculate a competitive index (CI) according to the following equation: $CI = (M_{out}/WT_{out})/(M_{in}/WT_{in})$, where M is the number of mutant cells and WT is the number of the wild-type cells in the inoculum (in) or in the recovered samples (out). The CI values were log transformed to allow even comparison between increases and decreases in competitive fitness. Significance was determined by Dunnett's *t* test, which is more conservative than individual pairwise *t* tests, using coinfections between *S.* Typhimurium ATCC 14028 and *S.* Typhimurium JS246, which is an isogenic derivative of *S.* Typhimurium ATCC 14028 and contains a neutral tetracycline resistance marker, as the control.

Expression of *sdiA-tnpR* in response to NaCl concentration. In order to test the effect of salt concentration on the expression of *sdiA-tnpR*, saltless LB was prepared, and NaCl was added to concentrations of 0.5 M (2.922 g/liter), 0.1 M (5.844 g/liter), 0.25 M (14.61 g/liter), and 0.5 M (29.22 g/liter) as either a liquid broth, soft agar (0.3% [wt/vol]), or solid agar (1.5% [wt/vol]). Overnight cultures were grown from glycerol stock in standard LB Lennox broth (5g NaCl/liter) with antibiotics. Prior to assays, cells were washed to remove tetracycline. Soft agar overlay plates were prepared by first allowing 10 ml of LB agar (1%) to solidify in a petri plate and then overlaying 7 ml of soft agar (0.3%). The prepared plates were inoculated with 10 μl of reporter culture via stab into the soft agar. For liquid culture assays, 5 ml of fresh LB broth was inoculated with a 1/10,000 dilution of washed reporter culture. All assays were incubated for 24 h at either 22°C or 37°C. Resolution of the reporter was then quantified as above.

Hemocyte assays. The colonization of oyster hemocytes was examined by adapting a method previously reported for *Vibrio splendidus* (33). A plasmid that constitutively expressed *gfp* was transformed into both wild-type *S.* Typhimurium ATCC 14028 and an *ssrB* mutant (MJW129) by electroporation. Green fluorescence was used to quantify hemocyte infection following either 2-h incubation with freshly drawn hemolymph or a 24-h exposure to live oysters. The 24-h exposure to live oysters was established in the same manner as RIVET reporter infections. For *in vitro* assays, hemolymph drawn from the pericardial cavity of freshly shucked oysters using a 22-gauge needle was mixed 50:1 with reporter culture and incubated at room temperature for 2 h. Fifty-microliter samples were prepared for microscopy, and 1-ml samples were used for FACS analysis. The reporter strains were prepared by diluting overnight cultures 1/100 into fresh LB and growing at 37°C for 2 h. The colonization of hemocytes was visualized via fluorescence microscopy (Olympus BX41). The proportion of colonized hemocytes was examined via flow cytometry in the UF ICBR core lab. Uninfected hemolymph and reporter culture were used as controls to set the sorting gates.

To determine if *Salmonella* organisms that had been internalized into hemocytes remained viable, gentamicin protection assays were performed using *S. enterica* MJW129, MHM51, and ATCC 14028 as a control. The concentration of hemocytes in freshly drawn hemolymph was determined using a Reichert Bright-Line hemocytometer. Overnight cultures of *Salmonella* were diluted 1/100 into LB prepared with 1/2-strength ASW and grown for 3 h at 37°C. The resulting culture was washed three times with 1/2-strength ASW, and 1 ml of a 1/1,000 dilution of *Salmonella* was mixed with 1 ml of hemolymph in 24-well plates at a *Salmonella*:hemocyte an approximate multiplicity of infection (MOI) of 5:1 and incubated at room temperature. Hemocytes were washed 3 times with 1/2-strength ASW to remove noninternalized *Salmonella* and then incubated in 1 ml of 1/2-strength ASW with 100 μg/ml gentamicin for another hour. Cells were washed 3 three times with 1/2-strength ASW to remove gentamicin and incubated in 1 ml 1/2-strength ASW for 30 min to allow for the possible digestion of internalized *Salmonella*. The hemocytes were lysed by incubating in 500 μl of 1% Triton X-100 for 15 min. A 10-fold dilution series was then plated onto LB and incubated overnight at 42°C. No growth was noted in hemocyte-only controls.

Quorum sensing reporter assays. Three complementary QS reporter assays were used: *Agrobacterium tumefaciens* NT4 pZLR4 (34), *Chromobacterium violaceum* CV026, and *C. violaceum* CV017 (35). *C. violaceum* CV026 and *A. tumefaciens* NT4 pZLR4 do not make their own QS signals and thus respond to exogenous agonists with short or long acyl side chains (respectively). *C. violaceum* CV017 produces its own C_6-HSL and is especially useful for detecting QS antagonists, including long-chain AHLs capable of interfering with the perception of the cognate AHL signal. Reporters were handled essentially as described previously (32, 36–38). Oysters were checked for native AHL activity by testing either organic extracts or direct overlays of dissected tissues. For tissue tests, the gills, labial palps, digestive glands, anus, and adductor muscle were dissected from freshly shucked oysters. The samples were placed into petri plates and set using medium with 1.5% agar. Oyster extracts were prepared by homogenizing oysters in a blender and then performing organic extractions with two equal volumes of acidified ethyl acetate. Phase separation occurred slowly due to the formation of gelled organic matter. The organic fraction was dried in a rotary evaporator at 45°C and stored at

−20°C. Extracts were resuspended in 200 μl methanol, and 50 μl was spotted to glass fiber discs and allowed to dry prior to the assay overlays. Production of AHLs by typical oyster microbiota was examined by collecting isolates grown from oyster homogenate plated onto either XLD, oyster agar, thiosulfate citrate bile salts sucrose agar (TCBS), or *Pseudomonas* selective isolation agar (PSIA) (39). Glycerol stocks of the saved colonies were plated directly to LB agar and grown for 48 h at 30°C. Plates were saved at 4°C until needed and prior to use were exposed to UV light for 10 min to inactivate the oyster-associated bacterial colonies. A 1/100 dilution of reporter culture in either LB 0.3% agar (*C. violaceum*) or M9 sucrose 1.5% agar (*A. tumefaciens*) was then poured over the prepared plates containing either tissue, extract, or colonies. The resulting plates were incubated at 30°C, and pictures were taken for comparison at 0 and 24 h.

RESULTS

Identification of AHL activity in oyster tissue and oyster-associated bacteria. The presence of activities capable of activating QS reporters was tested for with *A. tumefaciens* NT4 pZLR4 and *C. violaceum* CV026; *C. violaceum* CV017 (which makes its own AHLs) was used to detect AHL-inhibitory activity. Assays of oyster organic extracts with *Agrobacterium tumefaciens* NT4 pZLR4 were positive for AHL activity (Fig. 1A). Tests of dissected oyster tissue with a *Chromobacterium violaceum* CV026 overlay showed the strongest AHL activity within the gills and lower levels of activity in the digestive glands and labial palps (Fig. 1A and B). Because production of AHLs is autoregulated in a QS-dependent fashion, it is reasonable to hypothesize that microbial populations build up to quorate levels. Within digestive glands and labial palps, AHL-producing bacteria were either absent or present at prequorate levels of AHLs.

Of the 51 bacterial strains isolated from homogenized oysters, at least five were capable of inducing responses in *C. violaceum* CV026 and *A. tumefaciens* NT4 pZLR4, likely due to the production of AHL signals (Fig. 1C and E). Because *A. tumefaciens* NT4 pZRL4 assays involve the cleavage of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) by LacZ produced by the QS-responsive reporter *traG*::*lacZ*, the presence of β-galactosidase activity in the samples may confound interpretation of the results. Therefore, we carried out control experiments in which oyster isolates were tested on plates containing X-Gal, but in the absence *A. tumefaciens* NT4 pZRL4. As shown in Fig. 1F, none of the oyster isolates that triggered *A. tumefaciens* NT4 pZRL4 reporter were able to cleave X-Gal. Two oyster isolates inhibited AHL-mediated pigment production (but not growth) of *C. violaceum* CV017. These results indicate that bacteria capable of producing QS signals reside within oysters, and QS activities within oysters are readily extractable and detectable with common QS bioassays. While the chemical nature of these signals has not been elucidated, these studies provide a rationale for the hypothesis that the detection of QS signals from other bacteria by *Salmonella* may be important for its survival within oysters and their associated microbiota. This hypothesis was subsequently tested.

Identification of oyster-specific promoters using a *gfp* promoter probe library. To determine which *Salmonella* genes were differentially regulated during colonization of oysters, fluorescence-activated cell sorting (FACS) was carried out. FACS was consistently able to isolate a large population of *Salmonella* with active promoters (see Fig. S2 in the supplemental material). A total of 576 individual colonies selected as downregulated in LB liquid culture and upregulated during three subsequent passages through live oysters were recovered. They were then individually confirmed not to fluoresce on LB agar. Of those, 41 were selected based on strong differential fluorescence during growth in oyster homogenate versus LB. Sequencing of the selected colonies revealed 19 independent putative promoters (Table 2). Three of the sequences, *ssrB*, *rfaZ*, and *pagN*, identified regions that are internal to genes and oriented in the antisense direction, which could represent *cis*-encoded regulatory elements which have a role in post-transcriptional regulation.

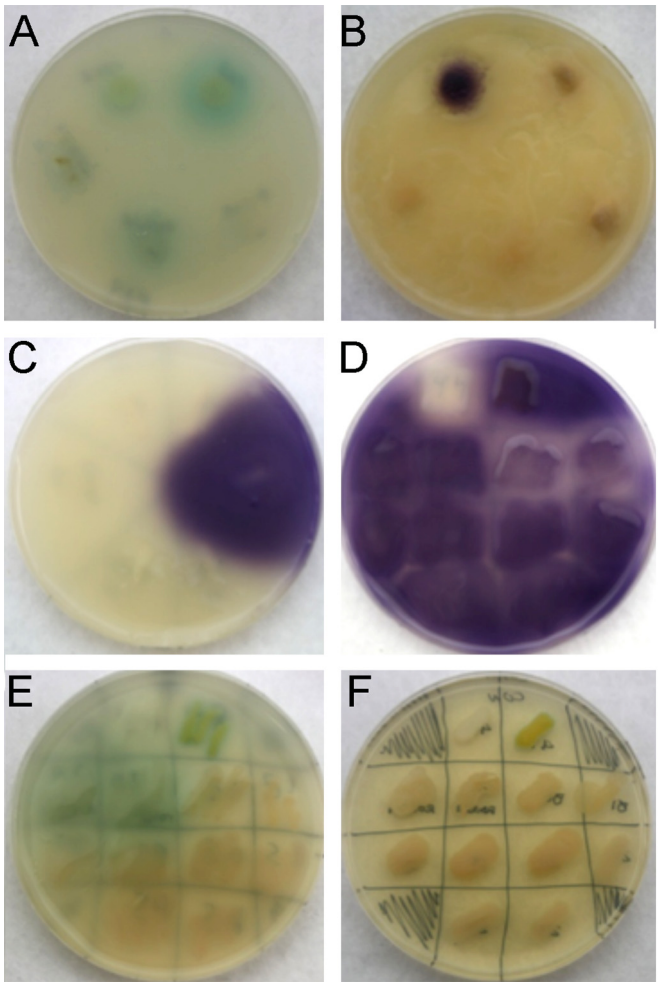

FIG 1 Detection of AHL activity using soft agar overlays of plates containing AHL reporter strains *Chromobacterium violaceum* CV026 and CV017 and *Agrobacterium tumefaciens* NT4 pZRL4. (A) Oyster organic extracts with the *A. tumefaciens* reporter overlay. Blue color indicates activation of the *traG-lacZ* AHL reporter by activities present in extracts. (B) Oyster tissue with *C. violaceum* CV026 overlay. Purple color indicates activation of the reporter. (C) Oyster-associated bacterial colonies with *C. violaceum* CV026 overlay showing activity in one isolate. (D) Oyster-associated bacterial colonies with *C. violaceum* CV017 overlay. Because *C. violaceum* CV017 makes its own AHLs and therefore violacein, the lack of purple color indicates strong inhibition of AHL quorum sensing by one colony and moderate inhibition by another two. (E) Oyster-associated bacterial colonies with *A. tumefaciens* reporter overlay. Two colonies show activity. (F) Control overlay without the *A. tumefaciens* reporter. The absence of blue color indicates no endogenous *lacZ* activity from these isolates in soft agar containing X-Gal.

Differential expression during *in vitro* RIVET assays. To verify results of the FACS and to quantify expression *in vivo*, single-

TABLE 2 Fragments recovered in the promoter-probe screen[a]

Category and putative function	Nearest gene	Probe orientation
General metabolism		
Glucogenesis (down regulated)	STM3500 (*pckA*)	
Folate biosynthesis	STM1198 (*pabC*)	
Thiosulfate Reductase (down regulated)	STM2065 (*phsA*)	
Glucokinase	STM2403 (*glk*)	
Ascorbate biosynthesis	STM3165 (*dkgA*)	
Pyrimidine biosynthesis (down regulated)	STM3968 (*udp*)	
Quinone oxidoreductase	STM4245 (*qor*)	
Cell envelope and stress response		
Adhesin/invasin	STM0306 (*pagN*)	
Osmotic stress	STM3067 (*yggB*)	
Predicted transport protein (down regulated)	STM3630 (*dppA*)	
O-antigen attachment	STM3715 (*rfaZ*)	
Peroxide resistance in biofilms	STM4379 (*yjfO*)	
Putative inner membrane protein	STM4596	
Regulatory		
SPI-2 regulator	STM1391 (*ssrB*)	
Carbon starvation induced regulator	STM2553 (*csiE*)	
Protein synthesis and degradation		
tRNA-dihydrouridine synthase B	STM3384 (*yhdG*)	
Unknown		
Hypothetical protein	STM1147	
Hypothetical protein (down regulated)	STM3689 (*yibL*)	
Predicted phosphatase (down regulated)	STM4026 (*yihX*)	

[a] Recovered fragments inducing promoterless *gfp* (and its orientation in relation to the *gfp* gene) are shown with white arrows, the nearest genes (listed in the second column) are shown with black arrows, and adjacent ORFs are shown with gray arrows.

copy chromosomal RIVET reporters were constructed for 18 of the 19 identified promoters. Several attempts were made to construct a *pckA-tnpR* reporter but were unsuccessful. *pckA* encodes phosphoenolpyruvate carboxykinase, which catalyzes the rate-controlling step of glucogenesis. Because of its central metabolic function and lack of a significant role in virulence during infection of mice (40), *pckA* was not examined further. Statistically significant differential regulation between the reporter in live oysters and on agar plates was detected in only four reporters, *udp* on LB agar, *qor* on 1/2-strength ASW soft agar, and *yibL* on LB and 1/2-strength ASW (Fig. 2A to C). These genes as well as *ssrB*, *yggB*, *yhdG* (*dusB*), *pagN*, *pabC*, *yibL*, *udp*, and *phsA* were selected as candidates for subsequent competition assays to determine whether they contribute to the survival in oysters. Despite detection of significant upregulation on 1/2-strength ASW soft agar, *qor* was not selected for subsequent competition assays as it encodes quinone oxidoreductase, an enzyme which plays a central role in electron transfer by catalyzing the oxidation of NADH to NAD^+. *qor* is known to be upregulated by RpoS-inducing conditions, including starvation and growth at 23°C, which likely accounts for the response on 1/2-strength ASW (41, 42).

Of the signaling-associated genes tested via RIVET, *csrB* and *luxS* were strongly expressed under all conditions (Fig. 2D). The *qseC* reporter was essentially not expressed under all conditions tested, regardless of the *luxS* background. *lsrG* had similar levels of expression in live oysters, oyster agar, and 1/2-strength ASW but had increased expression on LB. The expression of the *lsrG* reporter was essentially eliminated in the *luxS* mutant background, as has been shown in other systems (43). Only the *sdiA* reporter showed a significant difference in regulation, between live oysters and LB agar. Although *sdiA*'s downstream target, *srgE*, was also expressed on LB, the difference was not significant.

Competition assays. To follow up on the results of the RIVET assays and to test the hypothesis that *Salmonella* QS genes are involved in oyster colonization, fitness of the mutants with changes in QS and QS-regulated genes (*sdiA*, *rck*, *luxS*, *lsrACDBF*, and *qseC*) as well as the global regulator *sirA* and genes identified in a FACS screen (*pabC*, *udp*, *phsA*, *yhdG*, *yggB*, *pagN*, *ssrB*, and *yibL*) was tested in live oysters. Mutants were coinoculated in a 1:1 ratio with the parental wild-type strain *S.* Typhimurium ATCC 14028. To determine statistical significance of the pairwise fitness experiments, control experiments using tetracycline-tagged *S.* Typhimurium JS246 were carried out.

During competitive coinfection of live oysters, no signaling mutant showed a fitness phenotype (Fig. 3A). Of the eight mutants in the genes associated with the cloned regulatory region and selected from the RIVET screen, only an *ssrB* mutant was significantly less competitive than the wild type ($P < 0.0001$) (Fig. 3B). Because the *Salmonella* pathogenicity island 2 (SPI-2) *ssrB* is required for intracellular survival in vertebrate macrophages, the hypothesis that *Salmonella* colonizes oyster hemocytes, the immunocompetent cells responsible for pathogen recognition and killing via phagocytosis in bivalves (44), in an *ssrB*-dependent manner and uses SPI-2 as a conserved mechanism for persistence within oyster hemocytes was tested.

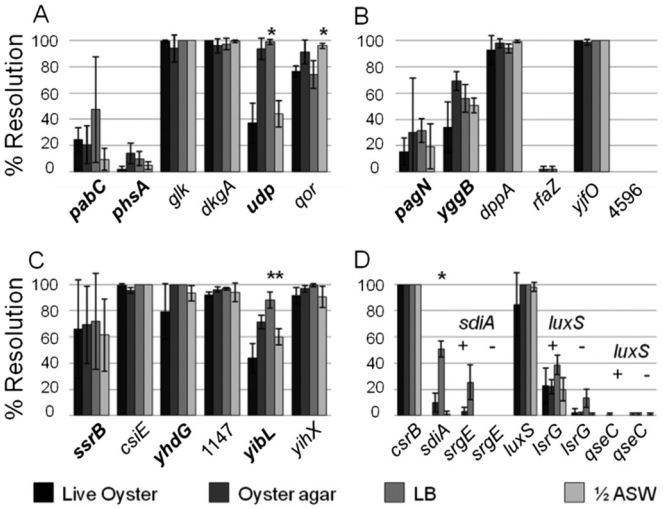

FIG 2 Resolution of *tnpR* RIVET reporters during 24-h incubation in live oysters or on oyster agar, LB agar, or 1/2-strength ASW soft agar at 22°C. RIVET provides a heritable market of promoter expression during growth *in vivo* by determining the percentage of the recovered population that has lost a selectable marker. Live oysters were incubated in 5 liters of 1/2-strength ASW containing 10^5 CFU/ml of reporter culture. A 10-μl sample of a 10^9-CFU/ml reporter culture was spot plated onto agar plates in parallel with the oyster infections. Error bars present standard deviations. *n* is 3 except for *upd*, where *n* is 4. An asterisk denotes a significant difference (Student's *t* test; $P \leq 0.05$) compared to the value for the same reporter in live oysters. The charts show genes picked up by the promoter probe screen and associated with general metabolism (A), cell envelope and stress response (B), and other processes (C). Reporters shown in bold were selected for competition assays. (D) Genes selected for study due to their association with quorum sensing.

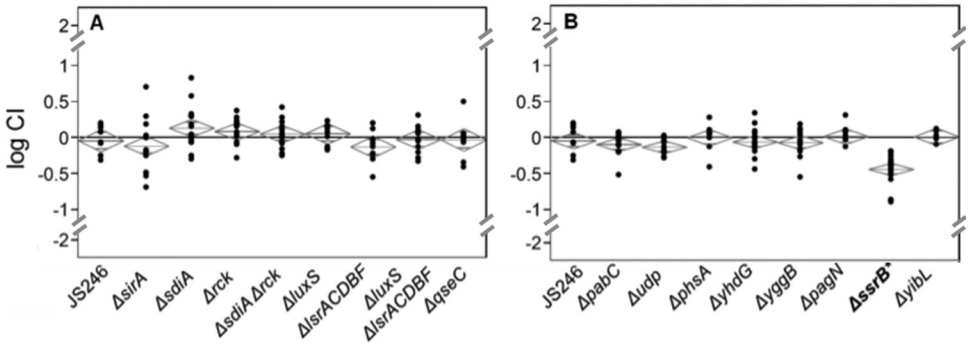

FIG 3 Competitive fitness of defined mutants versus wild-type *S. enterica* serovar Typhimurium ATCC 14028 in live oysters. (A) Quorum-sensing system mutants. (B) Mutants corresponding to the promoters identified by the GFP promoter probe library screen. The log CI (competitive index) was calculated as follows: $(M_{out}/WT_{out})/(M_{in}/WT_{in})$. The log transformation allows even comparison between increases and decreases in CI. Gray diamonds represent analysis of variance, where the middle line is the mean and the upper and lower lines show the 95% confidence intervals. Dunnett's *t* test was used to test for significance at a *P* value of <0.05, using coinfections between ATCC 14028 and the tetracycline-resistant *S. enterica* serovar Typhimurium strain JS246 as the control. The *ssrB* mutant was significantly less competitive than wild-type *Salmonella* ($P < 0.0001$). *n* is ≥ 8 for all mutants. Live oysters were incubated in 5 liters of 1/2-strength ASW containing 10^5 CFU/ml of reporter culture.

***ssrB* does not contribute to hemocyte invasion in oysters.** To test the hypothesis that *ssrB* is involved in interactions with hemocytes, the wild-type *S.* Typhimurium ATCC 14028 and an isogenic *ssrB* mutant MJW129 were tagged with the same fluorescent plasmid (pGFP-ON). FACS analysis of whole hemolymph drawn from oysters exposed to either *S.* Typhimurium ATCC 14028 pGFP-ON or an MJW129 (*ssrB*) pGFP-ON identified only a similarly small portion of the hemocyte population as GFP positive, suggesting that few hemocytes were colonized by either strain (Fig. 4). In order to simplify the infection, the same reporters were incubated with hemolymph harvested from live oysters for 2 h. Although FACS analysis identified a much higher percentage of the hemocyte population as GFP positive, the profiles for wild-type *Salmonella* and the *ssrB* mutant were again very similar (Fig. 5).

Subsequent examination by fluorescence microscopy showed a spectrum of colonization densities for the mutant and the wild type, from no associated bacteria to highly colonized individual hemocytes within each sample (Fig. 5; representative highly colonized hemocytes are shown). The hemocytes that were highly colonized by either strain also showed similar bacterial density, suggesting that the *ssrB* genotype did not impair *Salmonella*'s ability to enter hemocytes. The lack of an observed difference due to the *ssrB* genotype as well as the differences in the percentage of hemocytes colonized between the *in vitro* and *in vivo* infections indicate that if hemocyte invasion was responsible for the deficiency in competitive fitness of the *ssrB* mutant, then it has fundamentally different dynamics *in vivo*.

Because false-positive selection for GFP expression during FACS is theoretically possible if the GFP protein remained intact after internalized *Salmonella* was killed, gentamicin protection assays were used to quantify the percentage of cells that remained viable after internalization into hemocytes. The survival of both the *ssrB* mutant and an additional strain lacking all of SPI-2 (MHM51) were compared to wild-type *Salmonella* over 60 min after being internalized by hemocytes. Both the *ssrB* and SPI-2 mutants experienced lower survival than the wild type, but the difference was not significant (Fig. 6). In all cases, average survival was lower than 10%, indicating that oyster hemocytes were able to effectively degrade *Salmonella* under the test conditions.

Growth at 22°C and moderate NaCl concentrations do not impair *sdiA*. In the experiments whose results are presented in Fig. 2D, the AHL receptor *sdiA* was differentially regulated inside oysters and under *in vitro* conditions. Changes in temperature and salinity were hypothesized to be the conditions most likely to influence *sdiA* activation and SdiA activity. To test this hypothesis, expression of *sdiA-tnpR* and *srgE-tnpR* reporters was examined in soft agar with NaCl concentrations corresponding to freshwater (15 mM), human body fluids (150 mM), estuarine water (275 mM) and oceanic salt water (600 mM) at 22 and 37°C with or without AHLs. Growth at 37°C repressed *sdiA-tnpR* expression at all salinity levels. At 22°C, the *sdiA-tnpR* reporter was significantly more active at 150 mM and 275 mM (Fig. 7). As expected, the presence of AHL in the growth medium did not significantly alter *sdiA-tnpR* expression. Because *sdiA* was not expressed to an appreciable extent at 37°C, assays of the responses of the SdiA-dependent *srgE-tnpR* reporter were carried out only at 22°C.

In the absence of *sdiA* (with or without 3-oxo-C_6-HSL), at 22°C *srgE-tnpR* was essentially inactive (Fig. 8). With *sdiA*, the *srgE-tnpR* reporter was more active in the presence of AHLs at all salinities, as expected (36). Interestingly, resolution of *srgE-tnpR* was not significantly lower at 15 mM NaCl, as was the case for *sdiA*, suggesting that there was sufficient SdiA to activate *srgE* or that additional regulators could compensate for the lack of SdiA-mediated regulation. At 600 mM NaCl, *srgE-tnpR* resolution was significantly repressed regardless of the *sdiA* background. This effect could be due to the poor growth of *Salmonella* in general under these conditions.

DISCUSSION

The high prevalence of *Salmonella* in oysters raised questions about ecology of this pathogen outside its traditional hosts as well as the food safety consequences of its persistence in nontraditional hosts. Even though *Salmonella* is often recovered from live oysters, little is known about its persistence inside the animal or interactions with the native microbiota. *Salmonella* typically colonizes oysters at a density 1- to 2-log-fold below that of the inoculum (6, 11–15). A similar trend was observed in this study; the water in the oyster incubation tanks was inoculated with approximately 10^4 CFU/ml, and *Salmonella* was typically recovered from oysters at

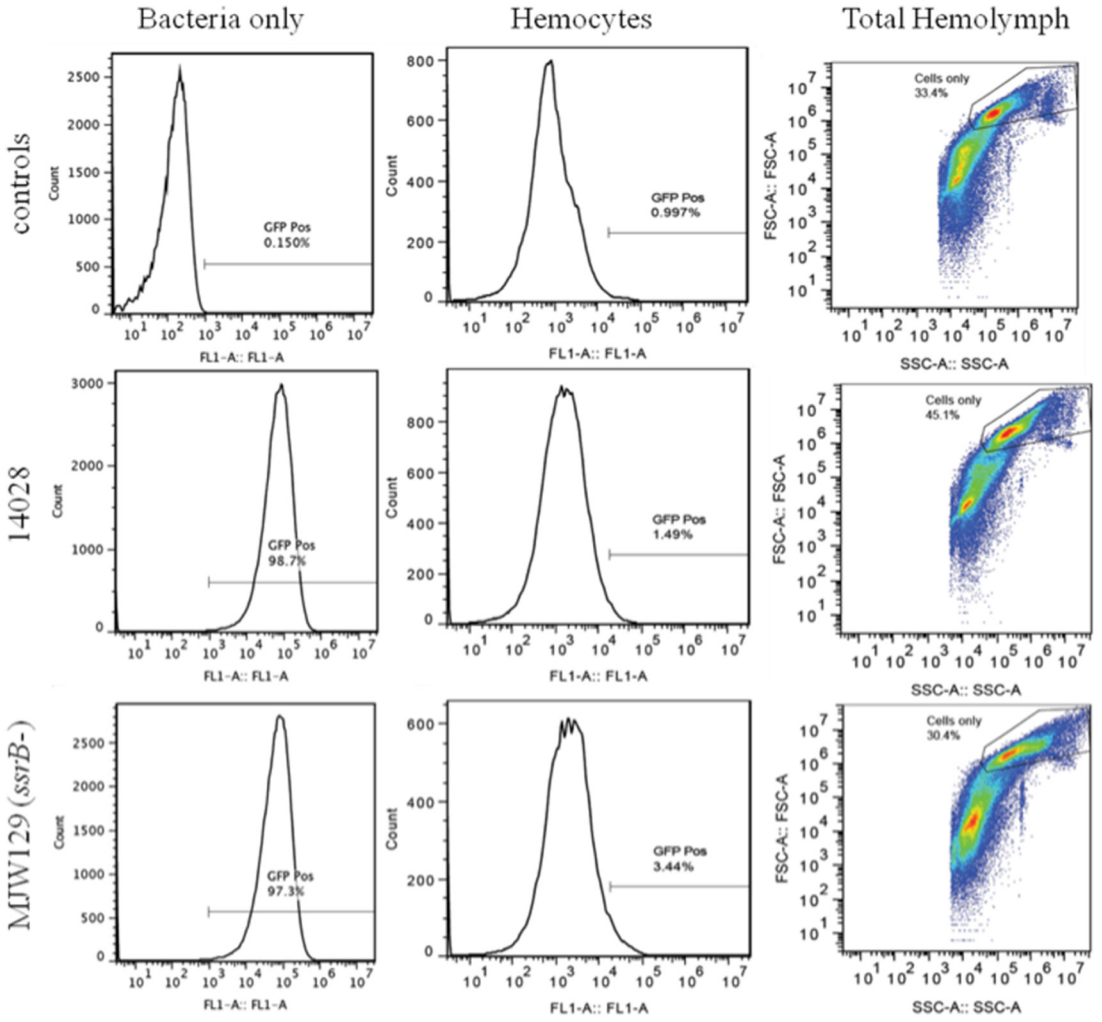

FIG 4 FACS analysis of hemocytes following a 24-h exposure of *gfp*-labeled *S. enterica* 14028 wild type or MJW129 (*ssrB* mutant) to live oysters. Live oysters were incubated in 5 liters of 1/2-strength ASW containing 10^5 CFU/ml of reporter culture. The bacteria-only plots show the population profile of the inoculum prior to oyster exposure. The bacteria-only control is unlabeled ATCC 14028. The hemocyte plots show the population profile of the cells selected as hemocytes by the sorting gates shown in the total hemolymph plots. The hemocyte control shows the analysis of hemolymph from an oyster which has not been exposed to *Salmonella*. All experiments repeated at least four times. Results are for representative samples. fsc, forward scatter; ssc, side scatter; FL1, fluorescence intensity.

10^2 to 10^3 CFU/g at 24 h postinoculation. Although long-term persistence of *Salmonella* in oysters is known to occur, it is unlikely that significant growth of *Salmonella* occurs within living oysters, as no study has observed a population expansion above the initial inoculation (6, 11–15).

The survival of *Salmonella* in oysters seems to be marked by a small population of initial colonizers which is able to persist but unable to expand and which slowly declines over time. Thus, it is likely that only a small portion of the total exposure population will be present in oysters postharvest, stressing the importance of understanding initial colonization factors in maintaining the microbial quality of live oysters. Therefore, with this study, we focused on the genes that could be involved in the initial colonization of oysters.

In an attempt to identify *Salmonella* genes that are involved in oyster colonization, we carried out a FACS screen as well as RIVET assays, followed by assays of defined mutants. Of the putative promoter regions identified by the screen, only one, *ssrB*, was linked to a competitive fitness phenotype in oysters. It is of note that the recovered *ssrB* fragment is in the antisense orientation. *ssrB* is part of the SsrAB two-component system that regulates genes encoded on *Salmonella* pathogenicity island 2 (SPI-2). SPI-2 is required for intracellular survival within vertebrate macrophages, and *ssrB* mutants are avirulent in mice (45, 46). We hypothesized that oyster hemocytes could be targeted by *Salmonella* in a similar manner as a strategy to establish a persistent infection. A similar strategy is utilized by *V. splendidus*, which specifically targets oyster hemocytes via agglutination to OmpU in order to establish intercellular infections (33). However, specific testing of this hypothesis by FACS using GFP-labeled wild type and isogenic mutants failed to demonstrate a difference in hemocyte colonization or a substantial population of hemocytes remaining colonized at 24 h in live oysters, suggesting that SPI-2-mediated colonization of hemocytes is not responsible for the *ssrB* fitness phenotype.

A previous study looked at the persistence of *Salmonella enterica* serovar Newport, the most common serotype isolated from

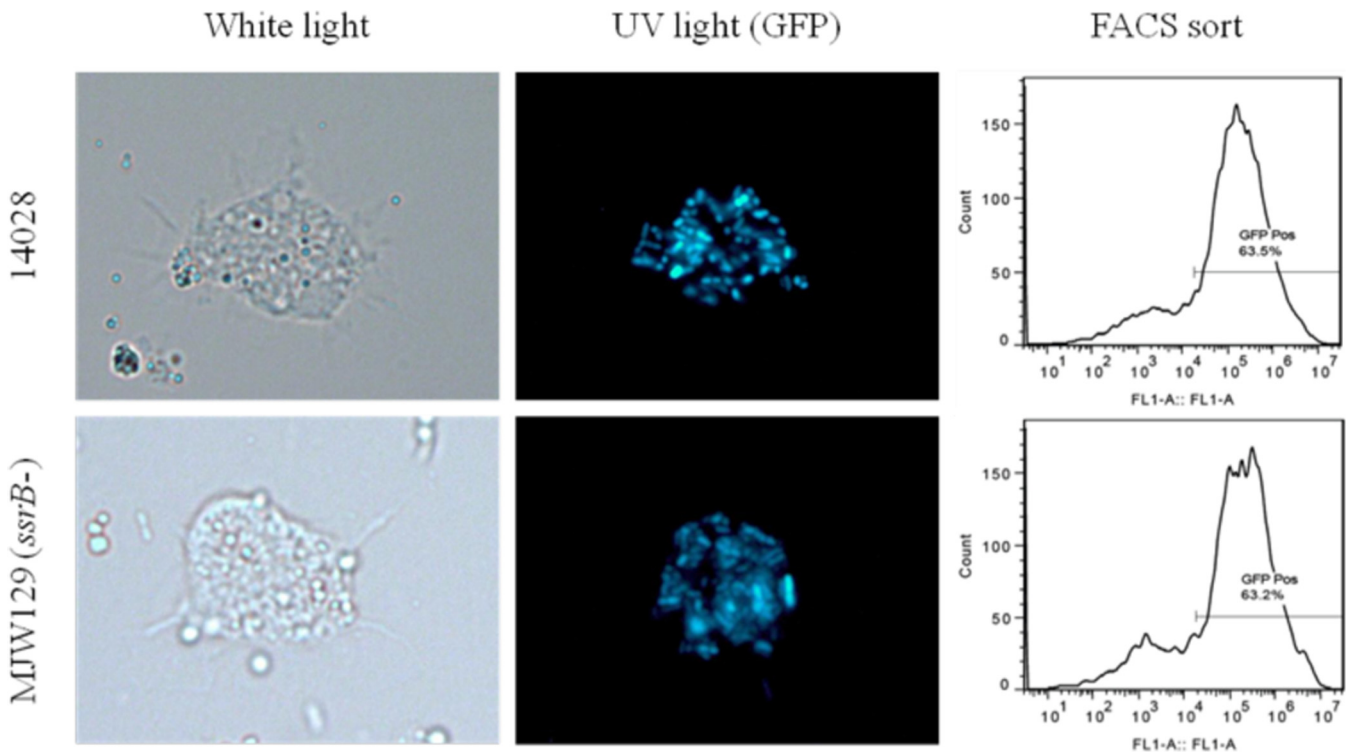

FIG 5 Colonization of hemocytes by *gfp*-labeled wild-type *S. enterica* ATCC 14028 and the mutant MJW129 (*ssrB*) during a 2-h exposure *in vitro*. Whole freshly drawn hemolymph was inoculated 50:1 with a 10^7 CFU/ml *Salmonella* reporter culture. Total volume per sample was 50 μl for microscopy samples and 1 ml for FACS samples. The white-light and UV-light images show the same hemocyte (imaged at 40×) for each *Salmonella* strain. The FACS displays the population distribution of hemocytes which contain *gfp*-expressing bacteria. At least 10,000 cells were counted. All experiments repeated at least four times. Results are for representative samples.

contaminated oysters, in live oysters over time (2, 20, 47, 48). After 15 days *Salmonella* is predominately located in the connective tissue, including the associated hemocytes (20). Because of the association with hemocytes, the long-term survival related to SPI-2 was specifically examined using an *ssaV* mutant deficient in the ability to secrete a SPI-2 effector protein (20). However, after 30 days postexposure, no discernible differences in population size were observed between the mutant and the wild-type *Salmonella*, indicating that SPI-2-mediated persistence did not affect the long-term persistence of *Salmonella* in live oysters (20). The differences observed in these two studies could be due to the serovar-level differences in the effects of SPI-2.

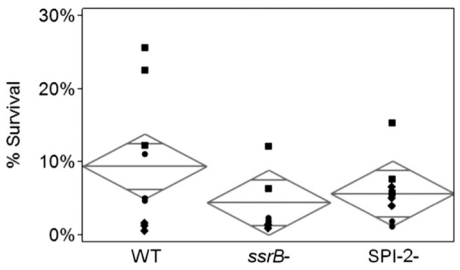

FIG 6 Gentamicin protection assay of oyster hemocytes exposed to *Salmonella* strains. The MOI varied between 5:1 and 1:1 in the independent experiments. Samples from the same independent experiment are represented by the same marker shape. There were no significant differences versus the wild-type control ATCC 14028, as determined by Dunnett's test. *ssrB*-, lacking *ssrB*; SPI-2-, lacking SPI-2.

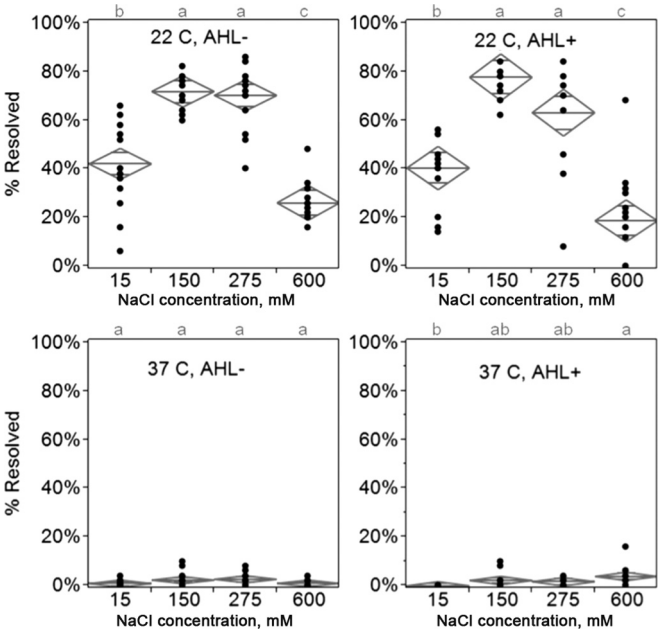

FIG 7 Resolution of the *sdiA-tnpR* RIVET reporter at either 22 or 37°C, on LB 0.3% agar containing 15, 150, 275, or 600 mM NaCl with (+) or without (-) 1 μM 3-oxo-C6 (AHL). Black dots represent individual samples from at least three independent experiments. *n* is ≥9 for all treatments. Diamonds show means and 95% confidence intervals. Lowercase gray letters indicate significance groups within each plot, as determined by the Tukey honestly significant difference (HSD) test.

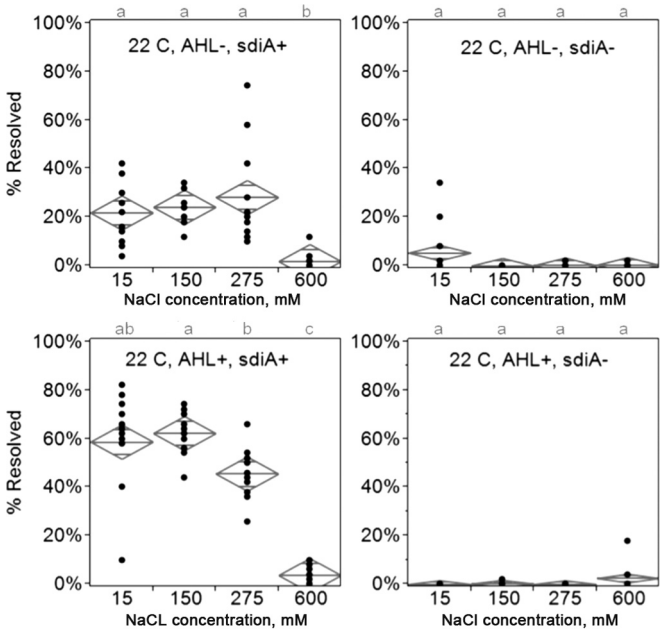

FIG 8 Resolution of the *srgE-tnpR* RIVET reporter in either an *sdiA*$^+$ or *sdiA* mutant (sdiA-) background at 22°C on LB 0.3% agar containing 15, 150, 275, or 600 mM NaCl with (+) or without (-) 1 μM 3-oxo-C6 (AHL). Black dots represent individual samples from at least three independent experiments. *n* is ≥9 for all treatments. Diamonds show mean and 95% confidence intervals. Lowercase gray letters indicate significance groups within each plot, as determined by the Tukey HSD test.

Although SPI-2 does not appear to play a role in the initial colonization or long-term persistence of *Salmonella* in oysters, *ssrB* could contribute to the fitness of *Salmonella* in oysters through regulatory mechanisms which are not currently understood. Studies show that *ssrB* can respond to multiple environmental signals, including acidic pH, low Mg^+ and PO_4^{3-} concentrations, and nutrient limitations (49–51). Extracts from *Bifidobacterium bifidum*, considered a probiotic strain, downregulated expression of an *ssrB-luxCDABE* reporter (52). An *ssrAB* mutant showed tissue- and inoculation method-specific differences in colonization during the infection of pigs (53). In addition, *ssrB* can alter gene expression through countering DNA silencing by H-NS, a global regulator which preferentially silences acquired DNA in *Salmonella* while allowing transcription of virulence factors (51).

Interestingly, our library screen identified a putative *cis*-encoded regulatory element within *ssrB*, which showed highly variable resolution in the RIVET assay, possibly indicating that this region has a regulatory role which could finely modulate expression of *ssrB* in response to environmental conditions altering downstream targets other than SPI-2. However, this hypothesis was not tested.

Our secondary hypothesis focused on the role of QS in the establishment of *Salmonella* within oyster-associated microbiota. Results of the AHL detection assays clearly show that QS activities are present within certain oyster tissues and that culturable members of the oyster microbiome can produce detectable QS signals *in vitro*. *Salmonella* SdiA is the receptor of AHLs produced by other bacteria, and although signal reception has been observed in several systems, its relevance to *in vivo* regulatory changes is less clear (32, 37, 38). The SdiA regulon in *Salmonella* is known to include *srgE* and the *rck* operon (23). SrgE is an effector secreted by a type 3 secretion system (54). The *rck* operon encodes resistance to complement killing and enables cellular invasion via a non-TTSS-dependent zipper mechanism (55). Both functions of *rck* appear to be mediated by cell surface properties (55). The regulation of both loci appears to be sensitive to environmental conditions (38, 55). However, inside oysters, *sdiA* and *srgE* were expressed at very low levels, while the corresponding mutants did not have reduced fitness within oysters.

Previous studies suggest that environmental conditions can interfere with expression of *sdiA* (32). Conditions within an oyster shell are strongly influenced by the external estuarine water. *In vitro* examination of the effect of temperature and salinity on expression of *sdiA-tnpR* showed stronger resolution at 22°C than at 37°C, indicating that SdiA is expressed at environmentally relevant temperatures, not solely the body temperature of vertebrate hosts. At 22°C, resolution of *sdiA-tnpR* was significantly higher on medium with 150 mM and 275 mM NaCl. This could indicate a salinity-specific response but could also be due to growth inhibition at the lowest and highest salinities. Decreased resolution of *sdiA-tnpR* on 1/2-strength ASW soft agar and oyster agar at 22°C, compared to LB agar, suggests that nutrient conditions may also affect the regulation of *sdiA*. The presence of AHL did not significantly alter *sdiA-tnpR* resolution, confirming that AHLs do not regulate *sdiA*. AHL-mediated expression of *srgE* at 22°C required *sdiA*, consistent with previous reports (36). Expression of *srgE-tnpR* at 22°C appears to decline at salinities above 150 mM but still demonstrates a response to AHLs in an SdiA-dependent manner at 275 mM. Taken together, these results suggest that the temperatures and salinities likely to be encountered by *Salmonella* when it colonizes an oyster host do not inhibit activation of *sdiA* or *srgE*. However, the lack of resolution of both *sdiA-tnpR* and *srgE-tnpR* in oysters, compared to on LB agar without AHLs, suggests either that the SdiA signal detection system is not active when oysters are being colonized or that the RIVET system employed here is not sufficiently sensitive. The absence of significant *in vivo* competitive fitness phenotypes for Δ*sdiA*, Δ*rck*, and Δ*sdiA* Δ*rck* mutants further supports the conclusion that SdiA-mediated detection of AHLs does not contribute to the colonization of oysters by *Salmonella*.

Interestingly, Rck is related to PagN, which was identified during the library screen but also showed no fitness phenotype during competitive coinfection of live oysters. A *pagN* mutation severely attenuates invasion of human epithelial cells and survival in mice, although the complete invasion mechanism is not currently understood (56–58).

A study which examined the fate of enteric pathogens exposed to live oysters found that most cells are shed intact in feces and remained viable, suggesting that oysters are able to prevent an active expansion of the *Salmonella* population via elimination from the digestive tract but do not necessarily induce significant pathogen mortality (59). This low mortality combined with a lack of growth, possibly due to limited nutrients, seems to place low selective pressure on the *Salmonella* population which remains in the oyster and could account for the small number of genes associated with a colonization phenotype during this study. Ultimately, *Salmonella* colonization of and persistence in oysters may be more a function of environmental adaptations.

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