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MEETING PRESENTATION

The Transcriptional Regulator HlyU Positively Regulates Expression of *exsA*, Leading to Type III Secretion System 1 Activation in *Vibrio parahaemolyticus*

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ABSTRACT *Vibrio parahaemolyticus* is a marine bacterium that is globally recognized as the leading cause of seafood-borne gastroenteritis. *V. parahaemolyticus* uses various toxins and two type 3 secretion systems (T3SS-1 and T3SS-2) to subvert host cells during infection. We previously determined that *V. parahaemolyticus* T3SS-1 activity is upregulated by increasing the expression level of the master regulator ExsA under specific growth conditions. In this study, we set out to identify *V. parahaemolyticus* genes responsible for linking environmental and growth signals to *exsA* gene expression. Using transposon mutagenesis in combination with a sensitive and quantitative luminescence screen, we identify HlyU and H-NS as two antagonistic regulatory proteins controlling the expression of *exsA* and, hence, T3SS-1 in *V. parahaemolyticus*. Disruption of *hns* leads to constitutive unregulated *exsA* gene expression, consistent with its known role in repressing *exsA* transcription. In contrast, genetic disruption of *hlyU* completely abrogated *exsA* expression and T3SS-1 activity. A *V. parahaemolyticus hlyU* null mutant was significantly deficient for T3SS-1-mediated host cell death during *in vitro* infection. DNA footprinting studies with purified HlyU revealed a 56-bp protected DNA region within the *exsA* promoter that contains an inverted repeat sequence. Genetic evidence suggests that HlyU acts as a derepressor, likely by displacing H-NS from the *exsA* promoter, leading to *exsA* gene expression and appropriately regulated T3SS-1 activity. Overall, the data implicate HlyU as a critical positive regulator of *V. parahaemolyticus* T3SS-1-mediated pathogenesis.

IMPORTANCE Many *Vibrio* species are zoonotic pathogens, infecting both animals and humans, resulting in significant morbidity and, in extreme cases, mortality. While many *Vibrio* species virulence genes are known, their associated regulation is often modestly understood. We set out to identify genetic factors of *V. parahaemolyticus* that are involved in activating *exsA* gene expression, a process linked to a type III secretion system involved in host cytotoxicity. We discover that *V. parahaemolyticus* employs a genetic regulatory switch involving H-NS and HlyU to control *exsA* promoter activity. While HlyU is a well-known positive regulator of *Vibrio* species virulence genes, this is the first report linking it to a transcriptional master regulator and type III secretion system paradigm.

KEYWORDS HlyU, *Vibrio*, enteric pathogens, gene regulation, transposons, type III secretion

Vibrio parahaemolyticus is a ubiquitous marine bacterium and is a leading cause of seafood-borne gastroenteritis globally (1). In brackish estuaries, these Gram-negative bacteria exist free-living in the water column, within marine sediments, and in commensal relationships with many bivalve shellfish, including oysters (2). After human consumption of contaminated shellfish, *V. parahaemolyticus* can cause vibriosis, leading

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to nausea, fever, diarrhea, and occasionally vomiting. Although generally self-limiting, *V. parahaemolyticus* causes significant morbidity during foodborne outbreaks (3). *V. parahaemolyticus* environmental isolates frequently exhibit genetic profiles; however, a clinically relevant pandemic *V. parahaemolyticus* serotype (O3:K6) has emerged, being isolated in patients around the world (1, 4). Additionally, specific *V. parahaemolyticus* strains that possess a toxin-antitoxin plasmid have emerged as significant shrimp pathogens, where major economic losses have occurred in aquaculture industries (5, 6). Thus, it appears these marine bacteria continue to evolve and are a serious threat to the seafood industry and human health.

V. parahaemolyticus virulence factors include hemolysins, toxins, and two type III secretion systems (T3SS), T3SS-1 and T3SS-2. The T3SS of pathogens acts as a molecular needle-like structure to deliver bacterial effector proteins into host cells (7). Sequence analysis of various *V. parahaemolyticus* clinical isolates has revealed that many contain both T3SSs (8, 9). T3SS-2 has primarily been linked to enterotoxicity (10, 11), whereas T3SS-1 has been linked to cellular disruption and rapid cytotoxicity (12–14). Many effectors are delivered via T3SS-1 into host cells and have targeted actions. VopQ contributes to rapid cell death during *V. parahaemolyticus* infection by interacting with lysosomal H^+ V-ATPases, causing lysosomal rupture and release of contents (15). Additionally, T3SS-1 effector proteins VopS, VopR, and VPA0450 have been implicated in immune evasion and actin rearrangement (13, 16). Other virulence mechanisms in *V. parahaemolyticus* include colonization factors (such as pili) and two type 6 secretion systems (T6SS) that likely aid in killing other bacteria or acting on macrophages during infection (17–19).

Over 40 genes within multiple genetic operons contribute to the formation of *V. parahaemolyticus* T3SS-1. The majority of these genes require the master regulator ExsA for their expression (20). ExsA and its orthologues in other bacteria are members of the AraC/XylR family of transcriptional regulators, many of which are implicated in bacterial pathogenesis mechanisms (21). In the cases of *Yersinia* species (LcrF), *Pseudomonas* species (ExsA), and enteropathogenic *Escherichia coli* (EPEC; GrlA), these regulators have been demonstrated to activate T3SS-related genes and have been linked to virulence phenotypes (22–25).

ExsA transcriptional activator roles are well documented, although it is not known how *V. parahaemolyticus* interprets environmental signals to activate *exsA* expression and thus initiate T3SS-1 biogenesis. In this study, we used a genome-wide transposon mutagenesis approach to identify genetic regulators of *exsA* expression. Through the design of a sensitive and quantitative luminescence screen, we identified a *cis*-acting genetic switch and implicate HlyU as a critical *V. parahaemolyticus* virulence determinant.

RESULTS

A sensitive functional screen identifies genes linked to *exsA* promoter activation. We initially generated a recombinant *V. parahaemolyticus* strain containing a promoterless bioluminescent reporter cassette (*luxCDABE*) transcriptionally fused to the *exsA* promoter region from *V. parahaemolyticus*. The p*exsA-luxCDABE* fusion was integrated into the *tdhS* locus of the *V. parahaemolyticus* RIMD2210633 genome (see Materials and Methods) (Fig. 1A). We chose the *tdhS* locus, as TdhS is not required for T3SS-1 activity (14). This strain (referred to as the Vp-*lux* strain) quantitatively reports the activity of the *exsA* promoter in real time by emitting bioluminescence, thereby allowing for sensitive *in situ* measurement of *exsA* promoter activation (Fig. 1B). The strain responded to magnesium and EGTA (T3SS-1-inducing conditions) by increasing *exsA* promoter activity as expected (Fig. 1B). A T3SS-1 protein secretion assay indicated that the Vp-*lux* strain secreted effectors similarly to wild-type *V. parahaemolyticus* (Fig. 1C).

Consequently, we employed a conjugative plasmid encoding a hyperactive transposase and its associated transposon (26) to mutagenize the Vp-*lux* strain. An optimized triparental mating approach resulted in the isolation of 4,302 stable transposants at an average transposition frequency of 1×10^{-6}. Using a high-throughput 96-well

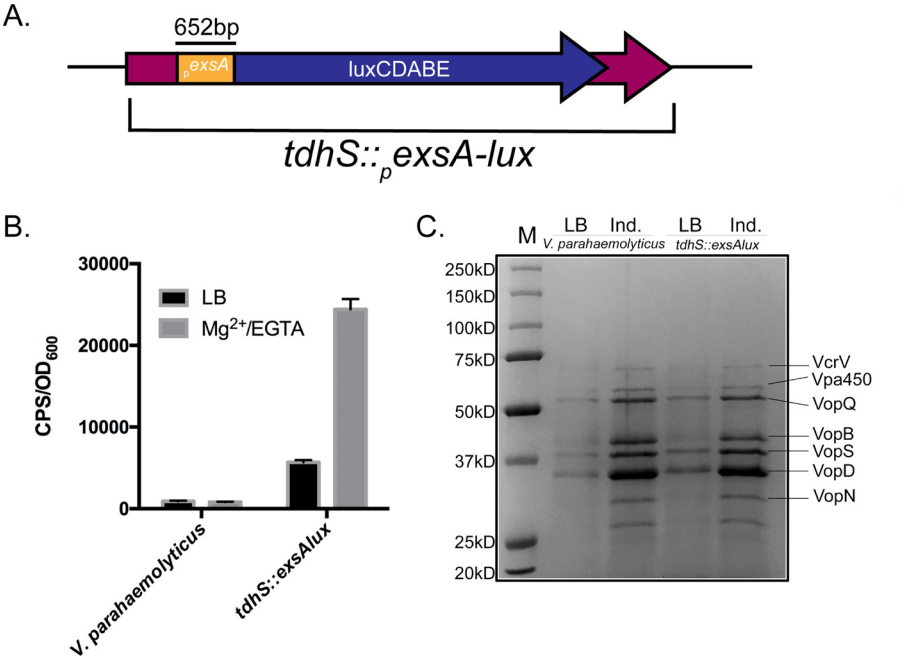

FIG 1 Integration of an *exsA* promoter-*luxCDABE* fusion into the *tdhS* locus (*tdhS*::*exsA-lux*) results in bioluminescence and a normal T3SS-1 secreted protein profile. (A) Schematic representation of the constructed *exsA* promoter-*luxCDABE* fusion inserted within the *tdhS* chromosomal locus. (B) Comparison of bioluminescence emitted by the indicated *V. parahaemolyticus* strains. Bacteria were cultured under T3SS-1-inducing conditions, sampled after 3 h of growth, and then assessed for light emission. cps/OD, counts per second divided by optical density (600 nm) of the cell suspension at time of collection. (C) Total secreted protein profiles of *V. parahaemolyticus* strains as determined by SDS-PAGE. Bacteria were grown in LB medium or LB supplemented with $MgSO_4$ and EGTA (induced [Ind.] for T3SS-1 activity). Labeled protein species have been previously identified by mass spectrometry analyses in wild-type *V. parahaemolyticus* (35). M, protein standard.

plate formatted assay, the transposants were screened for *exsA* promoter activity via bioluminescence. The Vp-*lux* strain served as a reference for defined normal growth and light emission. The screen identified a spectrum of mutants that exhibited altered bioluminescence levels compared to those of the parent Vp-*lux* strain (Fig. 2). Prior to investigating specific mutants, they were divided into 4 groups (or quadrants) to facilitate downstream analyses. This was achieved by establishing categories based on an optical density at 600 nm (OD_{600}) of 0.4 and a counts per second (cps) value of 1,000

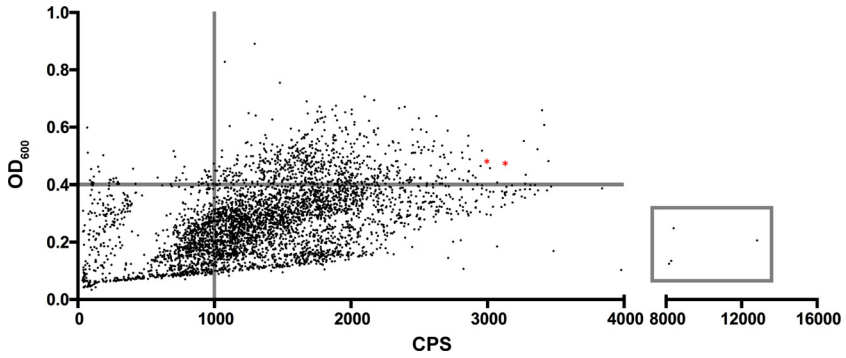

FIG 2 Bioluminescence scatterplot of Vp-*lux* Tn*5* insertional mutants. Transposon insertion mutants were assayed for bioluminescence emission. Each dot represents an individual mutant, and two representative controls (Vp-*lux* strain) are indicated as red asterisks. The vertical and horizontal bars represent boundaries used to categorize mutants based on light emission (*exsA* promoter activity) and cell growth, respectively. The box encompasses high-light-emitting mutants selected for further study. CPS, counts per second.

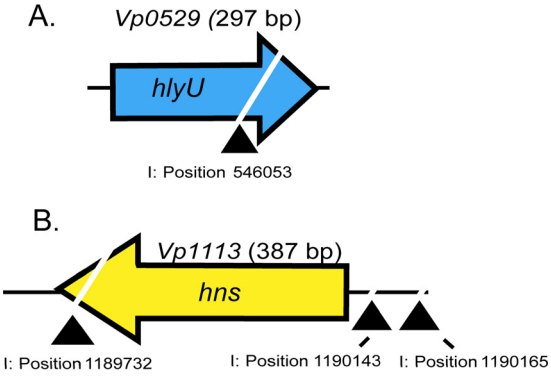

FIG 3 Schematic diagram of transposon insertion sites within selected genes of interest identified by luminescence screening of the Vp-*lux* Tn*5* mutant library. The triangles represent approximate transposon insertion sites with the approximate DNA base location in the *V. parahaemolyticus* genome (NBCI accession numbers NC_004603 and NC_004605; I, chromosome I).

and produced four quadrants (separate groups), including low-bioluminescence and normal growth, normal bioluminescence and normal growth, low bioluminescence and low growth, and high bioluminescence and any growth (Fig. 2).

Mutants that fell into the low-luminescence and normal-growth quadrant were further subjected to statistical narrowing. Using a binning procedure, mutants were subdivided into three categories by luminescence emission (measured in counts per second; low bioluminescence, 0 to 100 cps; low-moderate bioluminescence, 101 to 300 cps; and moderate bioluminescence, 301 to 1,000 cps). The means and standard deviations for each group were calculated. The means and standard deviations were calculated as 63.30 $\pm$ 10.68, 155.1 $\pm$ 22.32, and 542.7 $\pm$ 126.6, respectively. Six mutants whose cps reading was less than 1 standard deviation below the mean were then selected for further characterization in the low-bioluminescence group. Within the low-moderate and moderate bioluminescence groups, 3 and 4 mutants were selected for characterization, respectively. Lastly, 5 of the most bioluminescent mutants (Fig. 2, gray box) were also selected for characterization. Details of the selected mutants and statistical data are listed in the supplemental material (see Table S1 in the supplemental material).

Identification of genes with specific insertional transposons that alter *exsA* promoter activity. Using a genomic DNA marker retrieval protocol, we successfully identified chromosomal locations of transposition within mutants from the low-moderate and moderate light-emitting groups. From the low-moderate luminescence group linked to deficient *exsA* promoter activity, we mapped transposon insertions exclusively within *Vp0529* (Fig. 3A). Notably, for *Vp0529*, we identified three isolates with the exact same transposon insertion site. These likely represent a single clone that generationally expanded during the mutagenesis procedure or, alternatively, this chromosomal site is favored for transposon insertion. For the moderate bioluminescence group, we mapped two transposon insertion mutants, *Vpa0179* (*phoX*) and *Vp1473* (*merR* operon) (Fig. S1). Within the high-luminescence group linked to increased *exsA* promoter activity, 3 independent transposon insertions were mapped near or within *Vp1133* (*hns*) (Fig. 3B) and one insertion was mapped within *Vp1633* (RTX toxin) (Fig. S1).

Our initial efforts focused on *Vp0529* and *Vp1133*, since multiple independent mutant insertions were isolated from the *lux* reporter screen for these genes (Fig. 3) and therefore strongly implied a role in *exsA* regulation. *In silico* analysis of *Vp0529* using NCBI BLAST indicated sequence similarity to HlyU, a DNA binding protein of many *Vibrio* species that acts as a positive regulator of virulence genes (27–31). *Vp1133* encodes a histone-like nucleoid-structuring protein (H-NS) and has previously been implicated in *exsA* repression (32). To investigate this further, an assay that evaluates

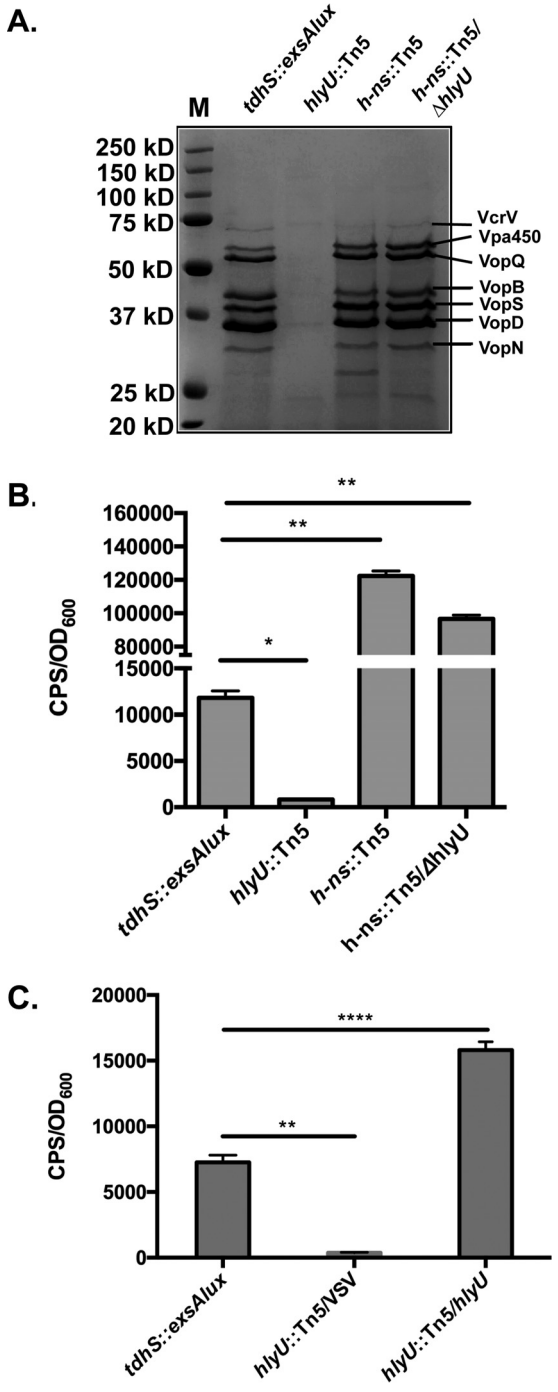

FIG 4 Protein secretion and *exsA* promoter activity assays with the Vp-*lux* (*tdhS*::*exsA-lux*) strain and specific transposon insertion mutants. (A) Total secreted proteins were collected from culture supernatants of the indicated Tn*5* insertion mutants and subjected to SDS-PAGE, followed by Coomassie blue staining. A protein ladder was included (M), and previously identified proteins are labeled. (B) Luciferase assays were conducted with the indicated Tn*5* insertion mutants and the *hns*::Tn*5* Δ*hlyU* double mutant (strain Vp-EL) ($n = 2$). The error bars indicate standard deviations from the mean values. A two-tailed *t* test was conducted to compare data sets. *, $P < 0.05$; **, $P < 0.005$. (C) Complementation of the *hlyU*::Tn*5* mutant with pVSV105 encoding *hlyU* was performed, followed by a luciferase assay. A two-tailed *t* test was used to compare data sets. **, $P < 0.005$; ****, $P < 0.0001$.

T3SS-1 protein secretion was performed. Critically, a marked decrease in many T3SS-1-dependent proteins was observed for the *Vp0529* Tn*5* insertion mutant compared to the Vp-*lux* parent strain (Fig. 4A). In contrast, the *Vp1133* mutant displayed secretion of many T3SS-1-dependent proteins, comparable to the parent strain.

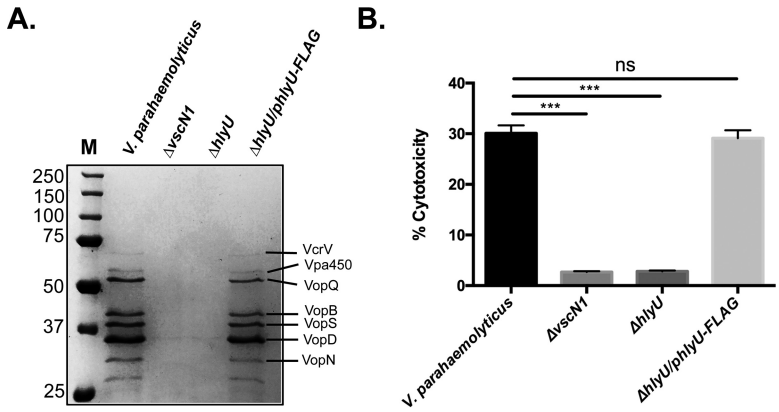

FIG 5 *V. parahaemolyticus* Δ*hlyU* mutant (strain hlyU1) is deficient for secretion of T3SS-1 proteins and exhibits reduced host cell cytotoxicity during infection. (A) Total secreted proteins were collected and subjected to SDS-PAGE, followed by Coomassie staining. A protein standard was included (M), and previously identified protein species are labeled. (B) Percent cytotoxicity was calculated after infection of HeLa cells with various strains by measuring released lactate dehydrogenase levels. All strains contained pVSV105 or p*hlyU*-FLAG as indicated. Statistical bars indicate standard deviations from the means, and statistical significance was determined by a paired two-tailed *t* test compared to the *V. parahaemolyticus* control. ***, $P < 0.001$. ns, not significant.

HlyU has previously been implicated as an H-NS derepressor, leading to *rtxA1* toxin expression in *V. vulnificus* (33). To investigate if HlyU acts as a derepressor of H-NS in *V. parahaemolyticus* or, alternatively, as a bona fide transcriptional activator, we deleted *hlyU* in the context of the *hns*::Tn*5* mutant (strain Vp-EL) and performed protein secretion and luciferase reporter assays. Vp-EL behaved similarly to the Δ*hns*::Tn*5* mutant, exhibiting T3SS-1 effectors and translocators along with enhanced luciferase activity (driven by the *exsA* promoter) (Fig. 4A and B). Therefore, in the absence of H-NS, HlyU is not required for T3SS-1 protein secretion or for *exsA* promoter activity. To confirm the role of HlyU for *exsA* promoter activity in cells expressing H-NS, we subjected the *hlyU*::Tn*5* mutant to genetic complementation. Enhanced restoration of luminescence was observed for the *hlyU* complemented strain that was significantly above the level of the Vp-*lux* parent strain (Fig. 4C).

***hlyU* null mutants are deficient for T3SS-1-dependent secretion and cytotoxic effects.** To validate the phenotypes measured in the *hlyU*::Tn*5* mutant and to rule out any effects due to transposon insertion, an *hlyU* null mutant derived from wild-type *V. parahaemolyticus* was generated (strain hlyU1) and tested for protein secretion. In complete agreement with the *hlyU*::Tn*5* mutant, T3SS-1 protein secretion for hlyU1 was deficient compared to that of wild-type *V. parahaemolyticus* and appeared similar to that of a Δ*vscN1* T3SS-1-deficient strain (Fig. 5A). Genetic complementation of strain hlyU1 with the wild-type *hlyU* coding region in *trans* fully restored T3SS-1 protein secretion.

To investigate the role of HlyU in the context of infection-like conditions, we conducted *in vitro* HeLa infections with *V. parahaemolyticus* strains followed by a lactate dehydrogenase (LDH) release cytotoxicity assay. As expected, wild-type *V. parahaemolyticus* resulted in rapid HeLa cytotoxicity in a *vscN1* (T3SS-1 ATPase)-dependent manner (Fig. 5B), confirming previous reports (34, 35). hlyU1 was significantly deficient in producing HeLa cytotoxicity (Fig. 5B), comparing closely to the low cytotoxicity levels observed for the Δ*vscN1* strain. Genetic complementation of hlyU1 restored HeLa cytotoxicity to wild-type *V. parahaemolyticus* levels, indicating that HlyU contributed to *V. parahaemolyticus* cytotoxic activity during *in vitro* infection.

Taken together, the protein secretion, promoter activation, and cytotoxicity data all indicated that HlyU was required for *exsA* promoter activation that supported expression of T3SS-1-related genes, leading to HeLa cell cytotoxicity during infection.

Purified HlyU binds upstream of *exsA*. HlyU is a member of the SmtB/ArsR family of regulator proteins that bind to DNA using a winged helix-turn-helix (wHTH) domain

structure (27). Based on the hlyU1 deficiency for T3SS-1-associated phenotypes, we set out to investigate whether HlyU binds to *V. parahaemolyticus* DNA. We hypothesized that DNA near the *exsA* promoter (located within our *lux* reporter constructs) should contain sequences that direct HlyU binding to DNA. To test this hypothesis, we overexpressed and purified a HlyU-His fusion protein (see Materials and Methods). Native HlyU is 98 amino acids in length (NCBI accession no. NP_796908.1) and has a predicted molecular mass of 11.2 kDa. Overexpressed HlyU-His appeared as an approximately 11-kDa protein by SDS-PAGE (Fig. S2). Electrophoresis mobility shift assays (EMSA) next were performed using an *exsA* promoter DNA fragment mixed with increasing amounts of purified HlyU protein. The *exsA* promoter DNA fragment demonstrated multiple shifts, with concentrated DNA species appearing with larger amounts of HlyU-His (Fig. 6A). For these larger HlyU-His amounts, Sypro red and SYBR green staining revealed protein-DNA complexes which appeared as tight bands coinciding with reduced mobility through the acrylamide gel matrix (Fig. S3). In contrast, no mobility shift was apparent for increasing amounts of bovine serum albumin (BSA), indicating that the *exsA* promoter region did not support nonspecific protein binding (Fig. 6A). Based on densitometric analyses of HlyU-bound *exsA* promoter DNA (Table S2), an apparent binding affinity ranging from 5 to 6.3 μM (95% confidence interval) was determined (Fig. S4). A nonspecific DNA fragment next was mixed with purified HlyU-His. While some minor shifts were observed at low HlyU levels (as seen by smearing), no major shifts or concentrated DNA species were apparent with increasing amounts of HlyU-His (Fig. 6B). These results suggest that small amounts of HlyU-His interacted with DNA weakly, and larger HlyU amounts specifically bound the *exsA* promoter region DNA, leading to homogenous protein-DNA complexes (see Discussion).

HlyU protects an inverted repeat sequence within the *exsA* promoter region from DNase I digestion. We set out to identify the DNA region bound by HlyU within the *exsA* promoter region. Fluorescently end-labeled *exsA* promoter DNA was mixed with purified HlyU-His and then treated with DNase I. The reaction mixtures were purified and then subjected to a DNA footprinting assay. As shown in Fig. 6C, a 56-bp region repeatedly and specifically produced low fluorescence signals indicative of an HlyU-protected region (undigested by DNase I). Sequence examination of this DNA region resulted in the identification of a perfect 7-base inverted repeat sequence, 5′-ATATTAG-3′, separated by an A/T tract of 14 bases. Furthermore, a 10-base direct-repeat palindromic sequence centered within the A/T tract is present (TAAATTAAAT) (Fig. 6C). Bioinformatic analyses indicated that this region of DNA is highly conserved in all current *V. parahaemolyticus* sequences in public databases and is located upstream of *exsA* in every case (data not shown).

DISCUSSION

We have identified a genetic regulatory switch that controls T3SS-1 gene expression by using a transposon mutagenesis approach coupled to a sensitive luciferase reporter screen. We set out to identify genes that promote *exsA* gene expression, which encodes the master transcriptional regulator of T3SS-1 in *V. parahaemolyticus*. The data suggest that *V. parahaemolyticus* HlyU acts as a derepressor by binding to DNA and relieving H-NS-mediated repression of *exsA* gene expression, thus leading to T3SS-1 activation. Critically, we demonstrated that HlyU is strictly required for *V. parahaemolyticus* to secrete specific effectors and induce rapid T3SS-1-mediated host cell cytotoxicity.

Within *Vibrio* species, it is well established that HlyU proteins act as global regulators of virulence genes. In *V. vulnificus*, HlyU functions to derepress the *rtxA1* toxin gene antagonizing H-NS binding (36). In this way HlyU acts as a derepressor, by removal of the H-NS-mediated repression, and not as a true RNA polymerase-recruiting transcriptional activator. In *V. cholerae*, HlyU acts to enhance promoter activity at genes associated with virulence. Insertional mutation of the *hlyU* gene in *V. cholerae* leads to a significant decrease in virulence within an infant mouse infection model, highlighting the importance of this gene for virulence (31). In *V. anguillarum*, HlyU controls the

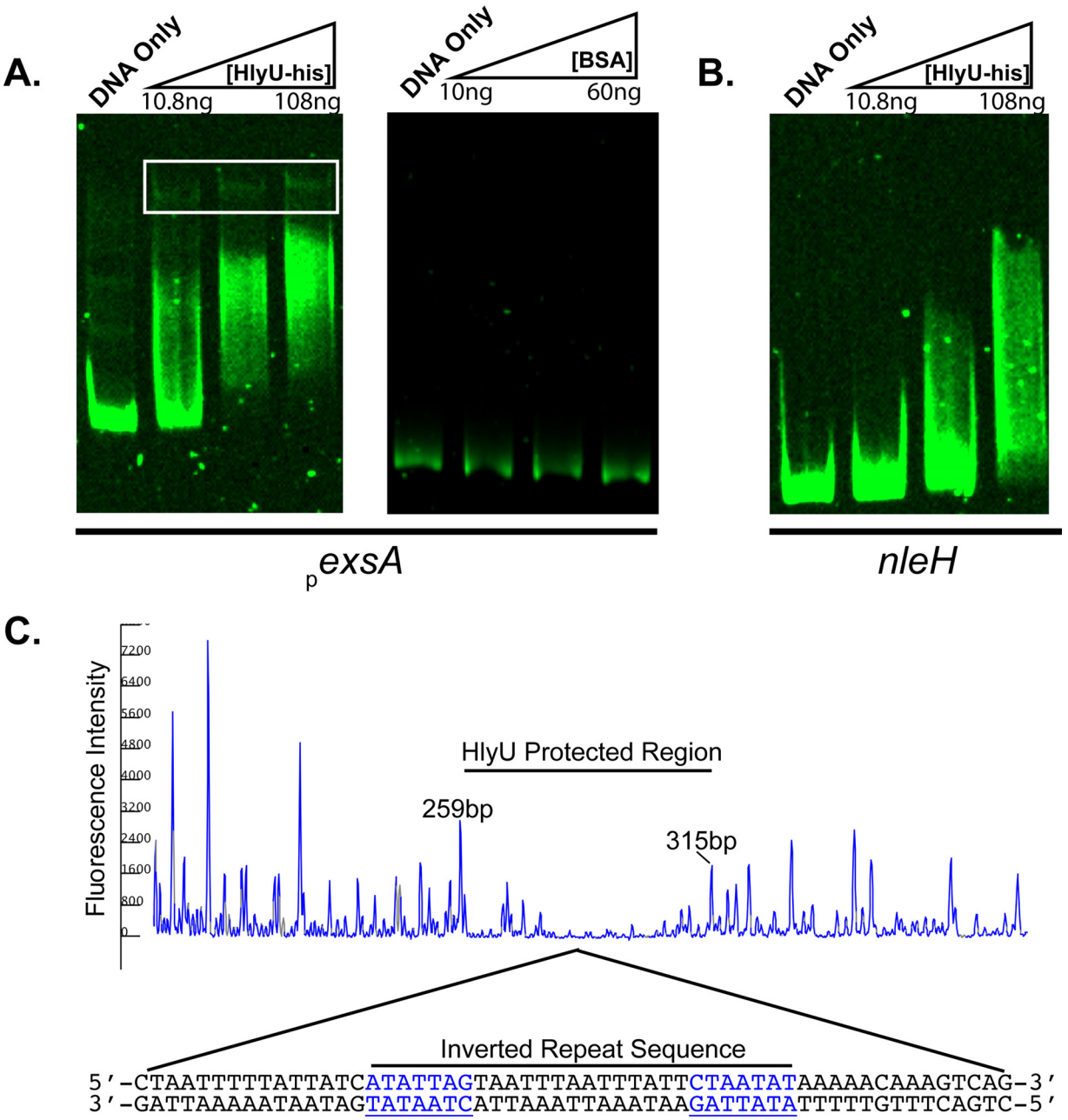

FIG 6 Electrophoresis mobility shift assay (EMSA) and DNA footprinting assays indicate HlyU binding to a region within the *exsA* promoter. (A) Six percent TBE-polyacrylamide gels were loaded with reaction mixtures containing *exsA* promoter DNA and increasing amounts of purified HlyU-His or bovine serum albumin (BSA). An *exsA* promoter DNA-only control was included as indicated. The white box indicates slow-migrating, concentrated DNA species. (B) A 6% TBE-polyacrylamide gel was loaded with reaction mixtures containing *nleH1* DNA and increasing amounts of purified HlyU-His. Following electrophoresis, gels were stained with SYBR green to specifically stain DNA. (C) A DNase I footprinting assay using purified HlyU-His was used to identify a DNA binding region for HlyU-His within the *exsA* promoter. The approximate base pair numbers of the HlyU-His footprint region are indicated (259 to 315 bp) and are based on capillary electrophoresis internal size standards. The DNA sequence associated with the identified HlyU protected region is displayed below the chromatogram. A 7-bp inverted repeat (labeled in blue) and separated by 14 bp is centrally located within the HlyU-His protected region.

expression of the *rtxACHBDE* and *vahI* gene clusters, whose protein products mediate the *V. anguillarum* hemolysin and cytotoxicity activities in fish (29).

Our data provide evidence that *V. parahaemolyticus* utilizes HlyU to positively regulate T3SS-1 expression, which is, to our knowledge, the first report linking this regulator to a type III secretion paradigm. Our transposon library approach separately and frequently identified both *hlyU* and *hns* as encoding protein regulators of *exsA*

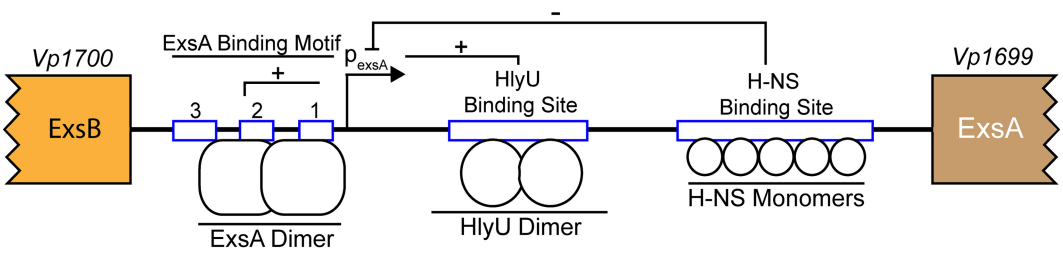

FIG 7 Schematic diagram of the intergenic region involved in *exsA* gene regulation. The ExsA binding motif and H-NS binding site have been previously identified. A putative HlyU binding site, as identified in this study, is shown. The ExsA binding motif has three box elements, each composed of conserved DNA sequences: 1, GC box; 2, A box; 3, TTAGN4TT. Protein-DNA interactions at specific sites within the *exsA* promoter region are shown. Plus and minus signs indicate positive and negative regulatory roles, respectively, on *exsA* promoter activity.

expression. H-NS has previously been implicated in T3SS-1 regulation in *V. parahaemolyticus* (32); therefore, we have independently confirmed those findings using a different approach. Our data suggest that HlyU is critical to derepress the *exsA* promoter by disrupting H-NS activity. In the absence of H-NS, HlyU is not required for *exsA* promoter activity (Fig. 4B). Instead, extremely elevated *exsA* promoter activity was observed in the absence of H-NS, suggesting that HlyU is not strictly required to activate the *exsA* promoter and likely serves a role to displace a negative regulator. Collectively, the data suggest that HlyU and H-NS form a genetic regulatory switch that serves to tightly control T3SS-1 gene expression in *V. parahaemolyticus*. Different genetic regulatory factors likely contribute to auxiliary *exsA* regulation, as our screen did identify other genes (see Table S1 in the supplemental material); however, the robustness of the phenotypes linked to *hlyU* and *hns* mutants implicate these respective genes in a central regulatory mechanism for *V. parahaemolyticus* T3SS-1 gene expression.

Negative regulation mediated by H-NS is a common paradigm for virulence genes, especially those found within genomic pathogenicity islands (37). It has been proposed that H-NS acts to protect the genome from foreign DNA that is acquired from various genetic transfer events. H-NS propensity to binding A/T-rich sequences likely serves to silence deleterious gene expression, which could stabilize the acquisition of new DNA while limiting detrimental fitness costs (38). Furthermore, pathogens capable of conditionally derepressing H-NS by expressing specialized DNA binding regulatory proteins would benefit from fine-tuning of virulence gene expression. Indeed, multiple examples of H-NS derepression by DNA binding proteins are known, including HilD (*Salmonella*), Ler (*E. coli*), and VirB (*Shigella*) (39–41). Many of these proteins bind at distinct DNA motifs and promote bending or alteration of DNA structure at specific genome sites. The consequence of DNA binding often locally displaces H-NS, thus supporting efficient promoter access and gene transcription (42). In the case of *V. parahaemolyticus*, we demonstrate that HlyU binds to DNA downstream of a previously reported *exsA* transcriptional start site and adjacent to where H-NS binds the DNA (43) (Fig. 7). The current data are not able to identify the exact regulatory mechanism underlying the HlyU–H-NS regulation of *exsA* expression; however, we hypothesize that under certain environmental or infection conditions HlyU is crucial to trigger *exsA* promoter activity.

HlyU protein sequences are highly conserved among *Vibrio* species (ranging from 81 to 100% identity) (27). Numerous structure-function studies have established that HlyU proteins form stable homodimers and possess a winged helix-turn-helix (wHTH) domain structure which is modeled to bind DNA within two major grooves (27, 44). Furthermore, HlyU dimerization is necessary for DNA binding activity in *V. cholerae*, and specific amino acids within HlyU contribute to DNA binding (27). These critical amino acids are conserved in all *V. parahaemolyticus* HlyU homologues within databases. We demonstrate here that *V. parahaemolyticus* HlyU binds to the DNA upstream of *exsA* using EMSA. Interestingly, we did observe various degrees of DNA shifts that were dependent on the amount of HlyU (Fig. 6A). A similar observation was previously

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Vibrio parahaemolyticus RIMD 2210633	Wild-type *V. parahaemolyticus*	8
Δ*vscN1* strain	*V. parahaemolyticus vscN1* null mutant	35
Vp-*lux* (*tdhS*::*exsA-lux*) strain	*V. parahaemolyticus* with the *exsA* promoter fused to *luxCDABE* gene cassette, integrated into chromosomal *tdhS* locus	This study
hlyU::Tn*5* strain	Vp-*lux* strain with transposon-disrupting *hlyU*	This study
h-ns::Tn*5* strain	Vp-*lux* strain with transposon-disrupting *hns*	This study
hlyU1	*V. parahaemolyticus* RIMD2210633 Δ*hlyU*	This study
Vp-EL	*hns*::Tn*5*-Δ*hlyU*	This study
DH5α	*Escherichia coli* cloning strain	Stratagene
DH5αλ*pir*	*E. coli* host for *oriR6K*-dependent plasmid replication	
BL21(λDE3)	*E. coli* host for protein overexpression studies	Novagen
pRE112	Suicide plasmid, R6K origin of replication	49
pJW15	p15A-based plasmid with promoterless *luxCDABE* gene cassette	50
pΔ*tdhS*	Deletion construct for *tdhS* within pRE112	This study
p*tdhS-exsAlux*	Integration construct to place promoter *exsA-luxCDABE* onto the *V. parahaemolyticus* chromosome within the *tdhS* locus	This study
pEVS104	Conjugative helper plasmid with mobilization machinery, used in triparental matings	26
pEVS170	Mini-Tn*5* transposon plasmid, erythromycin selection, R6K origin of replication	26
pVSV105	*Vibrio* shuttle vector with *lac* promoter and multiple cloning site, replication competent in *V. parahaemolyticus* and DH5αλ*pir*	45
pFLAG-CTC	Cloning plasmid to create C-terminal FLAG sequence fusions	Sigma
pFLAG-CTC-*hlyU*	*hlyU* fused with FLAG sequence	This study
p*hlyU*-FLAG	Expresses *hlyU* under the control of the *lac* promoter in pVSV105	This study

reported for *V. cholerae* HlyU (27). The current data cannot differentiate if the DNA shifts were due to (i) increased HlyU binding at multiple sites, creating larger DNA-protein complexes, or (ii) different physical conformations of DNA-protein complexes. DNase I footprinting assays repeatedly detected a single contiguous 56-bp stretch of protected *exsA* promoter DNA, suggesting that HlyU binds at or near this site. We identified a perfect 7-base inverted repeat located within this putative HlyU binding site. This feature is in agreement with *V. cholerae* HlyU, which also binds at an inverted repeat near its *rtx* operon; however, the sequence motif is different in each case. HlyU proteins from other *Vibrio* species bind at different repeat-type sequence motifs (27, 29); thus, it appears that some flexibility in sequence recognition exists. HlyU is considered a global virulence regulator, so it might be expected to recognize different sequences across the genome. Additional detailed studies will be required to determine sequence specificity requirements for HlyU binding to DNA.

In summary, we have identified a primary genetic switch composed of the DNA binding proteins HlyU and H-NS that serves to regulate T3SS-1 gene expression in *V. parahaemolyticus*. *V. parahaemolyticus* required HlyU to support the expression of ExsA, the master transcriptional regulator of multiple T3SS-1-associated genes. During infection-like conditions, HlyU was necessary for rapid host cell cytotoxicity mediated by T3SS-1. HlyU bound DNA near an inverted repeat located upstream of *exsA* which likely disrupts H-NS-mediated repression at this gene locus in *V. parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Vibrio parahaemolyticus* RIMD2210633 was grown in Luria broth (LB; L3522; Sigma) or LBS (10 g tryptone, 5 g yeast extract, 20 g NaCl, pH 8.0). All mutant derivatives described in this study were derived from *V. parahaemolyticus* RIMD2210633 (Table 1). All *Escherichia coli* strains were cultured in LB. The following antibiotics (Sigma) were used in growth medium as required: chloramphenicol (5 μg/ml and 30 μg/ml for *V. parahaemolyticus* and *E. coli*, respectively), erythromycin (10 μg/ml and 75 μg/ml for *V. parahaemolyticus* and *E. coli*, respectively), kanamycin (50 μg/ml), and ampicillin (100 μg/ml). *V. parahaemolyticus* was routinely grown at 30°C or 37°C aerobically with shaking at 200 rpm for 16 to 18 h. Agar (A5306; Sigma) was added to medium at 1.5% (wt/vol) for solid medium preparations. All plasmids used in this study are listed in Table 1.

Generation of a p*exsA-luxCDABE* transcriptional reporter in the *V. parahaemolyticus tdhS* allele. Primers NT393 and NT390 (synthesized by Integrated DNA Technologies [IDT]) were used in a PCR (with Phusion DNA polymerase; M0535S; New England BioLabs) with *V. parahaemolyticus* genomic DNA as the template. This approach amplified a contiguous DNA fragment encompassing upstream flanking se-

TABLE 2 List of oligonucleotides used in this study

Designation	Sequence (5′→3′)	Description or purpose
NT337	CCGAATTCAATCGGTTACATTTAATTAGCGC	*exsA* promoter forward
NT339	CCGGATCCCCGTTTCTGTGTTTAGTTGGCCTG	*exsA* promoter reverse
AL390	CGCTTAATTAATACATTGACCGGAGCTTG	*tdhS* mutant construction
AL391	CCTTAATTAAAGAGCGGTCATTCTGCTG	*tdhS* mutant construction
AL393	AGCTTACAGCTTGGTATGCCT	*tdhS* mutant construction
AL394	GTGGCTATGCACTGGCAGAT	*tdhS* mutant construction
NT394	AAGAGCTCACAGTATCCACTTACGTTGTTACG	Δ*hlyU* mutant construction
NT395	AACTCGAGTTCTTGTAGATTCATGTGTTGG	Δ*hlyU* mutant construction
NT396	AACTCGAGTGCGCAAACTGATCGCACAGACTG	Δ*hlyU* mutant construction
NT397	AAGGTACAAGCACGAGCAATCAACTCGC	Δ*hlyU* mutant construction
NT398	AACTCGAGTCAAGATACTTGGTTTAGTGAGC	*hlyU* genetic complementation
NT399	AAGGTACCGTTTGCGCAATAAAGACCGTGAAGG	*hlyU* genetic complementation
NT400	AGAATTCCATATGAATCTACAAGAAATGGAGAA	HlyU overexpression
NT401	AACTCGAGGTTTGCGCAATAAAGACCGTGAAGG	HlyU overexpression
NT402	6-FAMN/CCGAATTCAATCGGTTACATTTAATTAGCGC	*exsA* promoter forward-6-FAM
M13F	GTAAAACGACGGCCAGT	Sequencing primer
nleH1-F	GCGGTACCATGCTATCACCATCTTCTGTAAA	*nleH1* gene fragment
nleH1-R	GCACAATTGCCAATTTTACTTAATACCACACTAATAAG	*nleH1* gene fragment

quence of *tdhS* to a partial coding sequence of the gene. Similarly, primers NT391 and NT392 were used to amplify a distal *tdhS* coding sequence and downstream flanking DNA. These DNA fragments were digested with PacI, ligated together with T4 DNA ligase, and then used as a DNA template in a PCR with primers NT393 and NT392. The resulting DNA fragment was blunt-end cloned into the Eco53kI site of pRE112 to create pΔ*tdhS*. Primers NT337 and NT339 next were used with *V. parahaemolyticus* genomic DNA in a PCR to amplify the *V. parahaemolyticus exsA* promoter region. The resulting DNA product was subjected to restriction digestion with EcoRI and BamHI and then cloned upstream of the promoter-less *luxCDABE* gene cassette in pJW15. Finally, PacI digestion was used to excise the *exsA-luxCDABE* DNA fragment from pJW15, which was then cloned into the PacI site of pΔ*tdhS* to generate p*tdhS*::*exsAlux*. p*tdhS*::*exsAlux* then served as a chromosomal integration suicide construct via delivery into *V. parahaemolyticus* using triparental conjugal mating. Chromosomal integrants were selected with medium containing chloramphenicol and then streak purified. The integrants were then subjected to allelic exchange by SacB-mediated sucrose selection. Stable integrants within the *tdhS* allele were screened by PCR and then confirmed by Sanger sequencing. All oligonucleotides used in this study are listed in Table 2.

Generation of strain hlyU1 and construction of a plasmid for hlyU1 genetic complementation. A DNA fragment with the *hlyU* allele deleted was derived from *V. parahaemolyticus* genomic DNA by ligation PCR (primers are described in Table 2), cloned into pRE112, and then introduced into wild-type *V. parahaemolyticus*. Chloramphenicol selection for allelic exchange and then sucrose selection generated an *hlyU* null strain denoted the hlyU1 strain. An hlyU1 complementation construct was built using vibrio shuttle vector pVSV105 as a plasmid backbone (45). The *hlyU* DNA coding region was amplified by PCR using primers NT398 and NT399 and *V. parahaemolyticus* genomic DNA as the template. The *hlyU* DNA fragment was directionally cloned into pVSV105 as a KpnI and XhoI fragment. The resulting plasmid was transformed into *E. coli* DH5α*λpir* and then conjugated into the appropriate *V. parahaemolyticus* strains.

Mini-Tn*5* mutant library generation within the Vp-*lux* reporter strain. A mutagenesis procedure was followed, with minor modifications (26). Briefly, a conjugal mating on LBS agar allowed for the delivery of the plasposon pEVS170 into the Vp-*lux* strain. The mating mixture was plated onto selective LBS agar medium (pH 8.0) containing 10 μg/ml erythromycin and incubated at 22°C for 36 h. Transposants were then individually picked and grown on M9 minimal medium overnight. Finally, the transposants were grown overnight in LB (pH 8.0) supplemented with erythromycin and then frozen in 20% (vol/vol) glycerol in 96-well plate format.

Luciferase reporter library screen. Overnight cultures of the mini-Tn*5* mutant library in 96-well plates were grown at 37°C in 5.0% CO_2. A volume of 200 μl of LB medium supplemented with 5 mM EGTA and 15 mM $MgSO_4$ was accurately pipetted into each well of a sterile 96-well clear-bottom, white-walled plate (number 3632; Corning). A sterilized 96-metal-pin well replicator (V&P Scientific) was used to sample the overnight 96-well plate cultures, and then the replicator was used to inoculate each well of the 96-well plate supplemented with LB medium. These plates were incubated with shaking at 30°C and 250 rpm for 3.5 h. Luminometry (counts per second, read at 1 s per well) and OD_{600} endpoint readings were taken using a Victor X5 multilabel plate reader (PerkinElmer).

Statistical binning to categorize transposon mutants. To narrow mutants down to a reasonable number for genetic characterization, we undertook a statistical binning approach. All mutants that fell below 1,000 cps and above an OD_{600} of 0.4 were binned according to their counts per second into three categories: low glowers (less than 100 cps), low-moderate glowers (100 to 200 cps), and moderate glowers (200 to 1,000 cps). Statistical means and standard deviations were calculated for each group, and mutants that fell 1 standard deviation below the mean for each group were selected for characterization. These data are summarized in Table S1 in the supplemental material.

Genetic marker retrieval. Genomic DNA from selected *V. parahaemolyticus* transposants was isolated and restriction enzyme digested to completion using HhaI, followed by a heat inactivation of HhaI. The digested DNA (final concentration of approximately 40 ng/μl) was then treated with T4 DNA ligase overnight. Finally, the ligated DNA was transformed into *E. coli* DH5$\alpha\lambda pir$ using a standard procedure, and transformants containing self-replicating plasposons were selected on erythromycin LB agar medium. The plasposons were retrieved from the *E. coli* hosts using a standard miniprep procedure and were subjected to Sanger sequencing using the M13 forward sequencing primer. The sequencing data were compared to the RIMD2210633 reference *V. parahaemolyticus* genome to identify the genetic locus were transposition had occurred.

Protein secretion assays. T3SS-1 protein secretion assays were performed as previously described (35). Culture conditions that support T3SS-1 expression were a starting OD_{600} of 0.025 in LB supplemented with 15 mM $MgSO_4$ and 5 mM EGTA and a 4-h incubation at 30°C (250 rpm).

Cytotoxicity assays. HeLa cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco 11995) supplemented with fetal bovine serum, seeded in a sterile 24-well plate (number 3526; Costar) at a density of 10^5 cells/ml, and incubated for 16 h at 37°C in 5.0% CO_2 prior to infection. The HeLa cells were washed twice with 1 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4) before infection with selected *V. parahaemolyticus* strains. *V. parahaemolyticus* strains were cultured overnight in LB broth at 37°C and 200 rpm. The cultures were diluted in PBS and adjusted for cell number using OD_{600} measurement and then transferred to phenol red-free DMEM (Gibco 21063) (without serum), resulting in bacterial suspensions of $\sim 5 \times 10^5$ cells/ml. One milliliter of the relevant suspension was added to the appropriate wells of a HeLa cell-seeded 24-well plate for a multiplicity of infection (MOI) of approximately 5 (verified by viable plate counts). Uninoculated DMEM was added to wells containing the uninfected HeLa cells and the maximal LDH release condition controls. The 24-well plate was incubated for 4 h at 37°C and 5.0% CO_2. A cytotoxicity kit (88954; Pierce) was used according to the manufacturer's instructions. The following formula was used to calculate percent cytotoxicity: (experimental OD_{490} − uninfected OD_{490})/(maximal release OD_{490}) × 100.

Construction of a recombinant plasmid to overexpress and purify HlyU-His. Primers NT400 and NT401 were used in a PCR with *V. parahaemolyticus* genomic DNA to amplify the *hlyU* open reading frame without its stop codon. The resulting DNA fragment was digested with NdeI and XhoI and then cloned into the corresponding restriction sites within pET21a+ (Novagen), thus creating an in-frame fusion to a hexahistidine coding sequence (C-terminal His tag). The recombinant plasmid was initially transformed into DH5α and DNA sequence verified. Finally, the plasmid was moved into *E. coli* BL21(λDE3) for HlyU-His protein overexpression using a T7 inducible promoter system.

Overexpressed HlyU-His was purified from the soluble fraction of bacterial lysates using nickel-nitrilotriacetic acid (Ni-NTA)–agarose (Qiagen) and column chromatography as previously described (46). Extensively washed and purified HlyU-His was eluted from columns using an elution buffer (10 mM EDTA, 150 mM NaCl, 20 mM phosphate buffer).

EMSA. The electrophoresis mobility shift assay (EMSA) was performed as previously described (47), with a few minor modifications. Purified HlyU-His or BSA (B9000S; New England BioLabs) was mixed with a PCR-amplified *exsA* promoter DNA fragment in binding buffer (10 mM Tris [pH 7.5 at 20°C], 1 mM EDTA, 0.1 M KCl, 0.1 mM dithiothreitol, 5%, vol/vol, glycerol, 0.01 mg ml^{-1} BSA). A PCR-amplified *nleH1* gene fragment (derived from EPEC genomic DNA) served as an unrelated nonspecific DNA control. Six percent Tris-borate-EDTA (TBE)–polyacrylamide gels were prerun with 1.5 μl of 6× TBE loading dye (6 mM Tris, 0.6 mM EDTA, 30%, vol/vol, glycerol, 0.0006%, wt/vol, bromophenol blue, 0.0006%, wt/vol, xylene cyanol FF) and then loaded with the equilibrated protein-DNA samples. The gel was run for 4 h (100 V, 4°C) and then stained with SYBR green fluorescent DNA dye (Invitrogen) at a 1× concentration in TBE buffer and imaged using a VersaDoc MP5000 system (Bio-Rad). Protein staining and processing of TBE gels was performed using SYPRO Ruby Red (Bio-Rad) as previously described (48) and then imaged using a VersaDoc MP5000 system (Bio-Rad).

6-FAM DNase I footprinting assay. A 6-carboxyfluorescein (6-FAM)-end-labeled *exsA* promoter fragment was amplified by PCR using *V. parahaemolyticus* genomic DNA as the template with primers NT337 and NT402. Using the same EMSA binding conditions, the 6-FAM-labeled *exsA* promoter PCR product was mixed with purified HlyU-His or BSA and allowed to equilibrate for 30 min. Various amounts of DNase I (0.5 to 2 U) were added to the protein-DNA mixture, followed by immediate incubation at 37°C for 20 min. To stop DNase I activity, reaction mixtures were rapidly heated to 75°C for 10 min and then purified using a PCR purification kit (Qiagen). The samples were then subjected to capillary electrophoresis on an ABI-3730XL DNA Analyzer (Genome Québec Innovation Center). The chromatogram from this analysis was matched with a Sanger DNA sequencing reaction of the same *exsA* promoter DNA fragment. The HlyU-His protected region was identified by searching the chromatogram for a region with decreased 6-FAM fluorescence output. This experiment was repeated four times and included independent binding and DNase I digestion reactions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00653-17.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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