Control of the Type 3 Secretion System in *Vibrio harveyi* by Quorum Sensing through Repression of ExsA

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The type 3 secretion system (T3SS) genes of *Vibrio harveyi* are activated at low cell density and repressed at high cell density by quorum sensing (QS). Repression requires LuxR, the master transcriptional regulator of QS-controlled genes. Here, we determine the mechanism underlying the LuxR repression of the T3SS system. Using a fluorescence-based cell sorting approach, we isolated *V. harveyi* mutants that are unable to express T3SS genes at low cell density and identified two mutations in the *V. harveyi* exsB4 operon. While LuxR directly represses the expression of *exsB4*, complementation and epistasis analyses reveal that it is the repression of *exsA* expression, but not *exsB* expression, that is responsible for the QS-mediated repression of T3SS genes at high cell density. The present work further defines the genes in the *V. harveyi* QS regulon and elucidates a mechanism demonstrating how multiple regulators can be linked in series to direct the expression of QS target genes specifically at low or high cell density.

Bacterial pathogens must properly regulate the expression of virulence traits to successfully initiate and maintain disease. A type 3 secretion system (T3SS) is one such tightly controlled trait. T3SSs form needle-like pores through the inner and outer membranes of bacteria that deliver effector proteins from the cytoplasm of bacteria directly into their target eukaryotic host cells. Effector proteins have a variety of activities, including modifying host signaling and immune responses that result in disease progression (for a review, see reference 41).

*Vibrio harveyi*, a bioluminescent marine bacterium that infects marine animals, possesses a T3SS. In *V. harveyi*, the T3SS is regulated in a cell density-dependent manner by the process known as quorum sensing (QS) (24, 31, 43). QS is a mechanism of chemical communication that involves the production, secretion, and detection of signal molecules called autoinducers (AIs) (42). When bacteria are at low cell density, the concentrations of AIs are low, leading to the expression of behaviors that are important for survival as individuals. As a population of QS bacteria grows, the concentration of AIs increases proportionally. At a critical AI concentration, which corresponds to a particular population density, the population switches behaviors to those that are beneficial for survival as a group. QS regulates behaviors such as conjugation, bioluminescence, biofilm formation, and virulence factor production in many bacterial species (42).

*V. harveyi* produces and detects three AIs to monitor cell density (3, 10, 11, 25). Each AI is recognized by a cognate membrane-associated receptor (2, 3, 20, 25). At low cell density (i.e., in the absence of AIs), the receptors function as kinases and funnel phosphate into a phosphorelay pathway that ultimately leads to the phosphorylation of the response regulator LuxO (4, 19, 20). Phosphorylated LuxO (LuxO~P) activates the expression of genes encoding five regulatory small RNAs (sRNAs), termed the Qrr sRNAs, that repress the translation of luxR mRNA (27, 28, 39). LuxR is the master transcriptional regulator of QS-dependent genes (22, 24, 31, 38, 43). At high cell density, accumulated AIs inhibit the kinase activity of the receptors, switching their net activity to that of phosphatase. This event reverses the phosphate flow through the pathway, leading to the dephosphorylation of LuxO, the termination of the expression of the *qrr* genes, and the translation of the *luxR* mRNA. LuxR functions as both an activator and a repressor and is responsible for switching the gene expression patterns from those that underpin individual behavior to those appropriate for group behavior. In *V. harveyi*, more than 60 genes have been identified to be regulated by QS, but only 15 of them are directly controlled by LuxR (24, 31, 33, 43). This finding suggests that a hierarchy of gene regulation exists downstream of LuxR to control QS-dependent genes.

The T3SS of *V. harveyi* consists of four major structural operons and associated regulatory genes (24, 43). Three of the operons, denoted T3SS.1, T3SS.2, and T3SS.3, lie adjacent to one another on chromosome 1 of *V. harveyi* BB120 (ATTC BAA-1116; VIBHAR_01692 to VIBHAR_01712). This arrangement parallels that of *Vibrio parahaemolyticus* T3SS1, a close relative of *V. harveyi* (30, 47). *V. parahaemolyticus* also encodes a second T3SS (T3SS2) on chromosome 2 (30). Park et al. concluded that T3SS2 is not present in other *Vibrio* species by screening different species for homologous T3SS2 genes (32). In support of this conclusion, the recently completed *V. harveyi* BB120 genome reveals that none of the T3SS2 genes are present. Multiple studies have shown that the *V. harveyi* promoters...
driving the expression of the T3SS.I-T3SS.IV operons are expressed at low cell density and repressed by QS at high cell density (24, 31, 43). Furthermore, LuxR, the master transcriptional regulator of QS, is required for QS-mediated repression, because T3SS genes were not repressed at high cell density in a ΔluxR strain (24). Biochemical analyses to measure the secretion of the canonical effector protein VopD demonstrated that secretion occurs only at low cell density (24). In addition, the expression of T3SS.1, T3SS.2, and T3SS.3 are all required for the secretion of VopD, and T3SS.IV, while not yet tested, is presumed to be necessary as well. Finally, VopD secretion in V. para-halophilus is repressed by QS through the LuxR homolog, OpaR (24). However, the regulatory wiring linking LuxR and OpaR to the expression of the T3SS is not known.

In the present work, we characterize the mechanism by which QS, via LuxR, represses the expression of T3SS genes. Specifically we find that, at high cell density, LuxR functions indirectly to control T3SS gene expression by binding to a promoter upstream of the exsB operon, repressing the expression of both exsB and exsA. However, it is the repression of exsA (encoding a transcriptional activator) and not exsB (encoding a putative pilot protein) that is critical for the QS-mediated control of T3SS genes.

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TABLE 1. Strain list

| Strain   | Description                  | Reference |
|----------|------------------------------|-----------|
| BB120    | Wild-type V. harveyi         | 2         |
| KM83     | luxO(D47E)                   | 19        |
| JW507    | ΔluxR                       | This study|
| MR100    | ΔluxR, exsA::cat             | This study|
| MR100-flp| ΔluxR, ΔexsA                 | This study|
| JW510-flp| ΔluxR, ΔexsB::cat            | This study|
| JW510    | ΔluxR, ΔexsB                 | This study|
| JM401    | luxO(D47E), vopN::mini-MucA  | This study|
| KM470    | luxO(D47E), vspC::mini-MucA  | This study|
| KM476    | luxO(D47E), vspB::mini-MucA  | This study|
| JW103    | ΔluxR, ΔexsB::Tn5, C6        | This study|
| JW104    | ΔluxR, ΔexsB::Tn5, E10       | This study|
| BH421    | luxA::Tn5                    | This study|

recognition sites and subsequently cloned into the BamHI restriction site of pLAFR2. The exsA gene was amplified to contain a 5’ EcoRI site and 3’ BamHI site. The 3’ primer was designed to incorporate the FLAG sequence DYKD-DDDK-stop at the terminus of the exsA open reading frame (ORF) to confirm the expression of ExsA upon the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The ExsA-FLAG-encoding fragment was cloned into the EcoRI and BamHI sites of the overexpression vector pEVS143 (43). GFP transcriptional fusions were constructed in pCMW1 (43) by cloning into the Spel and Sall restriction sites. The exsB-lux fusion was cloned into the SpeI and BamHI sites of pBBRlux (23), while the exsA-lux fusion was cloned into the BamHI site of pBBRlux. All plasmid constructions were confirmed by sequencing. Primer sequences are available upon request.

Genetic screen to identify QS-controlled regulators of the T3SS. To find putative mutants of the V. harveyi luxR mutant JW507 was performed using the suicide vector pRL272 (26). Ten thousand kanamycin-resistant colonies were pooled. Plasmid pCMW209-cat contains a 319-bp V. harveyi genomic fragment containing the promoter region of the T3SS.IV operon in a transcriptional fusion to gfp. This fragment was isolated in a previous genetic screen as a QS-repressed promoter. In addition, the kanamycin resistance gene of the original isolate, pCMW209 (45), was replaced with a chloramphenicol acetyltransferase (cat) gene to generate pCMW209-cat. The expression of the fusion is repressed 50-fold by LuxR (43). pCMW209-cat was introduced into the above-described Tn5 mutant pools by conjugation. Exconjugant colonies were pooled and grown for 20 to 24 h at 30°C in LM medium containing chloramphenicol. This time point was selected because it provides a homogenous population of cells expressing high levels of GFP. Cells from these cultures were diluted 1:50 in LB and spotted on a medium containing chloramphenicol flow cytometer (Becton Dickinson), and the lowest 1% of gfp-expressing transformants was isolated. The selected cells were individually transferred to 96-well plates (Costar 3595; Corning) containing 200 μL of LB medium with chloramphenicol. The plates were agitated for 20 24 h at 30°C, and green fluorescent protein (GFP) production from each well was measured on a 1420 Victor2 multilabel counter (Wallac). Mutants exhibiting low gfp expression were collected and grown in fresh medium, and gfp expression was verified by flow cytometry. This strategy identified 21 candidates expressing low gfp. The pCMW209-cat plasmid was isolated from each candidate mutant pool, reintroduced into strain JW507 to confirm that no spontaneous mutations had occurred on the reporter plasmid to cause reductions in gfp expression.

Genomic DNA was isolated from confirmed candidate mutants using a DNeasy blood and tissue kit (Qiagen), and the DNA was digested separately with BamHI, MluI, or EcoRV. Resulting fragments were circularized by ligation and introduced into E. coli S17-1pir by electroporation. Plasmids containing the transposon and flanking V. parahaemolyticus DNA were selected on LB medium with kanamycin and sequenced. Sequence analysis was performed using Vector NTI suite 10.11 (InforMax Invitrogen Software Package), the Institute for Genomic Research (TIGR) V. parahaemolyticus database (http://www.tigr.org), and NCBI BLAST-X (http://www.ncbi.nlm.nih.gov/BLAST/).

Fluorescence, bioluminescence, and qPCR measurements. All gene expression measurements were performed between 20 and 24 h of growth. When the original screen was performed, gfp expression was measured using a BD FACSAria (Becton Dickinson) flow cytometer in overnight cultures grown for 20 to 24 h in triplicate (settings: forward scatter [log] voltage, 400; side scatter [log] voltage, 505; and fluorescein isothiocyanate [log] voltage, 626) or, as mentioned, using a 1420 Victor2 multilabel counter (Wallac). For the analysis of bioluminescence expression, strains were grown with shaking in 150 μL from a 1/150 dilution of an overnight culture in Costar 3903 microtiter plates. Bioluminescence was determined on a Molecular Devices SpectraMax M5 microplate spectrophotometer system. For quantitative real-time PCR (qPCR), DNA was isolated from V. harveyi strains grown to an optical density at 600 nm (OD600) of 1.0 using the RNeasy mini kit (Qiagen). DNA was quantified on a NanoDrop ND-1000 spectrophotometer ( NanoDrop Technologies). Samples were treated with DNAse I (Roche) in reaction mixtures containing 10× PCR buffer (ABI) and 25 μM MgCl2 (ABI). cDNA was generated in 100-μl reaction mixtures containing 1 μg of RNA, 5× first-strand buffer (Invitrogen), 100 μM dithiothreitol (DTT) (Invitrogen), 10 μM deoxynucleoside triphosphates (dNTPs) (ABI), random hexamers (Roche), and SuperScript II reverse transcriptase (Invitrogen). Reactions were conducted in a thermocycler for 10 min at 25°C, for 50 min at 42°C, and lastly for 10 min at 72°C. To control for genomic DNA contamination, identical reactions were performed in the absence of reverse transcriptase. Real-time PCR was performed on an ABI Prism 7900HT sequence detection system. Reactions were carried out in 384-well optical reaction plates (ABI) in quadruplicate with 2× SYBR green mix (ABI). Ten microliters was loaded into each well using a Beckman Coulter Biomek FX machine. Real-time PCR primers were designed using Primer Express 2.0 (ABI Software). http://aem.asm.org/ Downloaded on April 26, 2019 by guest
mRNA was used as an endogenous loading control for the reactions. Relative mRNA levels using ΔΔCT relative quantification were determined by the RQ Manager, version 1.2, software program.

Electrophoretic mobility shift assays (EMSAs). LuxR was purified with the IMPACT protein purification system (NEB) as previously described (43). DNA probes for gel mobility shift analyses were generated using 5'-6-carboxyfluorescein-5'-(and-6)-FAM; mixed isomers) 5'-tagged fluorescent primers in a standard PCR and purified using the Zymoclean gel DNA recovery kit (Zymo Research). Each probe (10 nM, 5 μl total volume) was combined with 1 μl of 1 μM of poly(dl-dC) and the indicated amount of LuxR (0 to 1.000 nM) in a final volume of 20 μl at 30°C for 15 min. Thus, every reaction mixture contained a total of 14 μl of the buffer that was used to purify LuxR (20 mM Tris [pH 7.5], 1 mM EDTA, 10 mM NaCl, and 0.1 mM DTT with 20% glycerol [43]). Mobility shifts were performed on 5% Tris-acetate-EDTA (TAE)-polyacrylamide gels and visualized using a Storm 860 imaging system (Molecular Dynamics).

Examination of operon structure of exsA4 and 5'-rapid amplification of cDNA ends (RACE) analysis. To determine if exsB and exsA are cotranscribed, RNA was prepared from a ΔluxR V. harveyi mutant grown to an OD600 of 1.0 using the RNaseay protocol (Qiagen) and treated with Turbo DNase (Invitrogen). One μg of RNA was converted to cDNA using iScript (Bio-Rad). A control reaction that did not contain reverse transcriptase was prepared from a wild type of BB120 genomic DNA, the reverse transcription-negative control, and the cDNA samples using a standard PCR with Phire DNA polymerase (NEB) for 35 cycles. The reaction product was amplified and purified using the Zymoclean gel DNA recovery kit (Zymo Research). A 10 μM of the buffer that was used to purify LuxR (20 mM Tris [pH 7.5], 1 mM EDTA, 10 mM NaCl, and 0.1 mM DTT with 20% glycerol [43]). Mobility shifts were performed on 5% Tris-acetate-EDTA (TAE)-polyacrylamide gels and visualized using a Storm 860 imaging system (Molecular Dynamics).

RESULTS

Identifying the regulatory pathway connecting QS to expression of T3SS genes. To determine the mechanism by which QS controls T3SS gene expression in V. harveyi, we first examined whether LuxR is a direct regulator of the T3SS promoters. To do this, we performed EMSAs with purified LuxR and fluorescently labeled probes encoding the upstream regions of each of the four T3SS operons. LuxR showed no binding to these promoter regions, suggesting that LuxR controls T3SS genes by an indirect mechanism (data not shown). This result is consistent with our previous finding that LuxR does not directly bind to the promoter region upstream of the T3SS IV operon encoded in the plasmid pCMW209 (43).

To identify regulatory elements linking LuxR to the repression of T3SS gene expression, we developed a fluorescence-activated cell sorting (FACS)-based screen. The plasmid pCMW209-cat, encoding a gfp transcriptional fusion to the exsD promoter (this region is shown in Fig. 1A), was used as the readout for T3SS expression. pCMW209-cat is highly expressed in a ΔluxR mutant, and gfp expression is repressed 50-fold in strains that are WT for luxR (43). We hypothesized that LuxR regulates T3SS in V. harveyi through the repression of an activator protein or the induction of a repressor protein. To examine the first possibility, we performed mutagenesis of a ΔluxR mutant and screened for mutants that showed the loss of the expression of pCMW209-cat.

Mutants containing random Tn5 insertions generated in a ΔluxR V. harveyi strain were pooled, pCMW209-cat was introduced, and mutants exhibiting reduced gfp expression relative to that of pCMW209-cat in the parent ΔluxR strain were isolated using FACS. The locations of the transposon insertions in 21 such mutants were identified. Fifteen of these insertion mutants were siblings, so five unique transposon mutants were identified. A single unique insertion was located in the following genes: VIBHAR_00301, a putative hydrodase; VIBHAR_06142, ribokinase; and VIBHAR_05400 and VIBHAR_05860, hypothetical genes. Two independent insertions, C6 and E10, were located in the gene VIBHAR_01694 (Fig. 1A and B). VIBHAR_01694 encodes a homolog of the Pseudomonas aeruginosa exsB gene (21) (also named yscW or virG in Yersinia enterocolitica). In Y. enterocolitica, virG encodes a pilot lipoprotein required for the efficient assembly of the T3SS ring complex (9). VirG partners with YscC, a secretin, that oligomerizes to form the upper basal body of the Y. enterocolitica T3SS needle complex (9). VirG partners with YscC, a secretin, that oligomerizes to form the upper basal body of the Y. enterocolitica T3SS needle complex (9). VIBHAR_01694 (here referred to as ExsB) and VirG from Y. enterocolitica share only 16.9% amino acid identity, making it unclear if they are functionally similar.

Mutation of exsB alters T3SS gene expression. exsB is located approximately 2 kb upstream of the T3SS IV operon of V. harveyi (Fig. 1A). Importantly, in the exsB::Tn5 mutants C6 and E10, exsD-gfp transcription was assayed in trans from the

FIG. 1. Mutations that alter T3SS gene expression at low cell density. (A) A map of the region upstream of the T3SS IV operon (the first two genes of which, exsD and nosA, are shown). Numbers under each gene refer to the ViBHar gene designation in the sequence of the V. harveyi BB120 genome, while the names correspond to the closest homolog of each gene. The percentages under each gene refer to the percent identity of the predicted protein with the Vibrio para-haemolyticus homolog. The locations of the two insertion mutations in exsB, C6 Tn5 and E10 Tn5, described in the text, are shown. The region containing the promoter of the T3SS IV operon cloned into pCMW209 and pCMW209-cat is shown as an arrow above the genes and is denoted 209. The locations of the two promoters identified in this study, P1 and P2, are indicated by arrows. (B) exsD-gfp expression from pCMW209-cat (bars 2 to 4) and from pCMW209 (bar 5) in the ΔluxR V. harveyi mutant and in three ΔluxR, exsB mutants are shown. Error bars indicate standard deviations.
bioluminescence/OD600. The white bars in A and B show background described for panel A. Relative luminescence units (RLUs) indicate luxational fusion of the T3SS.I promoter to strain, so we conclude that the expression level of the T3SS.I cat/H9004 to that of the gfp T3SS.IV promoters to operon. (A) Transcriptional fusions of the T3SS.II, T3SS.III, and plasmid pCMW209-cat. Therefore, the reduction in gfp expression from the T3SS.IV operon, or if this mutation re-

FIG. 2. Mutation of exsB decreases the expression of every T3SS operon. (A) Transcriptional fusions of the T3SS.II, T3SS.III, and T3SS.IV promoters to gfp were measured in ΔluxR V. harveyi (black bars) and in the ΔluxR, exsB::cat mutant (gray bars). (B) A transcriptional fusion of the T3SS.I promoter to lux was measured in the strains described for panel A. Relative luminescence units (RLUs) indicate bioluminescence/OD600. The white bars in A and B show background fluorescence and the bioluminescence of V. harveyi, respectively. Error bars indicate standard deviations.

We wondered if the exsB::cat mutation specifically reduced expression from the T3SS.IV operon, or if this mutation reduced the expression of all of the T3SS operons in V. harveyi. To determine this, each T3SS operon promoter was cloned as a gfp transcriptional fusion into plasmid pCMW1. The expression of each of the fusions was measured in the ΔluxR and ΔluxR, exsB::cat mutants (Fig. 2). No gfp expression could be detected for the T3SS.I promoter fusion in either mutant strain, so we conclude that the expression level of the T3SS.I promoter is below the limit of detection for gfp in our system under all conditions. However, gfp expression from the T3SS.II and T3SS.III fusions was reduced 6- and 2-fold, respectively, in the ΔluxR, exsB::cat double mutant compared to that of the ΔluxR parent (Fig. 2A). This result is similar to what we observe for the effect of exsB::cat on the expression of the T3SS.IV promoter (Fig. 1B, 2A). To determine if the promoter of the T3SS.I operon also was repressed in the ΔluxR, exsB::cat mutant, we constructed a transcriptional fusion of this promoter to the lux operon in the pBBRlux plasmid (23). Luciferase provides a much more sensitive reporter for gene expression, and indeed the transcription of the T3SS.I operon was detectable from this reporter. Similarly to the results observed for the promoters of the T3SS.II-T3SS.IV operons, the expression of the T3SS.I operon was reduced 3-fold in an exsB::cat mutant (Fig. 2B). In each case, the fold reduction in the expression of the T3SS.I-III promoters in the exsB::cat mutant was less than what we observed for the T3SS.IV promoter. The T3SS.IV promoter has the highest levels of the expression of the four T3SS operons in V. harveyi, and thus it apparently provides a larger dynamic range of regulation. The importance of these differences in expression levels is not known. In summary, the mutation of exsB leads to an overall loss of T3SS gene expression from all four T3SS operons in V. harveyi.

QS regulates exsB expression. If exsB is part of the regulatory pathway linking QS to T3SS gene expression, we predict that exsB also is regulated by QS. To determine if this is the case, quantitative PCR was used to measure the expression of exsB in four V. harveyi strains: the wild type grown to high cell density, the ΔluxO mutant that is locked into the high-cell-density state, and the luxO(D47E) and ΔluxR mutants that are locked into the low-cell-density state. The ΔluxR mutant is locked at high cell density because it does not express qrr, so LuxR is constitutively produced. In contrast, the luxO(D47E) allele locks the cells into the low-cell-density state because LuxOD47E mimics phospho-LuxO, leading to the constitutive activation of the qrr genes and the repression of LuxR independent of cell density. Finally, in the ΔluxR strain, the cells are locked at low cell density because there is no LuxR to promote the high-cell-density gene expression pattern (reviewed in reference 42). Our results are shown in Fig. 3 and indeed are analogous to what we observe for the T3SS genes; the expression of exsB is 50-fold higher in the low-cell-density-locked strains than in the wild-type and ΔluxO high-cell-density strains.

Secretion is not required for T3SS gene expression. In T3SSs of many bacteria, functional secretion is intimately tied to the expression of genes required for the assembly of the T3SS. Gen-
generally, secretion activates T3SS gene expression at the level of transcription via the secretion of regulatory proteins through the T3SS apparatus (reviewed in reference 8). Based on these earlier findings, we hypothesized that the decrease in T3SS gene expression that occurs in the exsB::cat mutant could be due to a defect in secretion through the T3SS apparatus, leading to the failure to export a negative regulatory protein. If so, we reasoned that other mutations that abolish secretion would similarly repress the transcription of the promoters controlling the expression of the T3SS genes. To test this idea, exsD-gfp production from pCMW209 was analyzed in previously identified transposon insertion mutants in the T3SS.1 operon (vopB::mini-MuacZ), the T3SS.2 operon (vopN::mini-MuacZ), and the T3SS.3 operon (vscP::mini-MuacZ). Our previous results indicate that each of these mutations results in defective type three secretion in V. harveyi (24). To activate transcription from the promoters of the T3SS operons in these mutants, the luxO(D47E) allele was incorporated into the chromosome of each transposon mutant. As mentioned above, the luxO(D47E) allele constitutively locks the cells into the low-cell-density state and causes the maximal expression of T3SS genes. Consequently, the expression of exsD-gfp from pCMW209 is high in the luxO(D47E) strain (Fig. 4). Transposon mutations in the T3SS.1, T3SS.2, or T3SS.3 operon in combination with luxO(D47E) caused no decrease in exsD-gfp expression (Fig. 4), in contrast to what we observed for the exsB::cat mutation (Fig. 1, 2). Thus, we conclude that the loss of T3SS gene expression in the exsB mutant is not due to a secretion defect.

**Mutation of exsB affects exsA.** To determine if the loss of ExsB production is solely responsible for reduced T3SS expression, we attempted to complement the exsB::cat mutation with the exsB gene and a region 500 bp upstream of its translation start site cloned into the pLAFR2 plasmid (Fig. 5A). The complementation plasmid was introduced into the ΔluxR, exsB::cat mutant strain containing pCMW209-cat (exsD-gfp), and gfp expression was measured. As previously described, the expression of exsD-gfp is high in the ΔluxR strain and low in the ΔluxR, exsB::cat strain. However, the introduction of the exsB gene alone did not restore the transcription of exsD-gfp in the ΔluxR, exsB::cat mutant (Fig. 5A). Using quantitative PCR, we confirmed that exsB mRNA was produced in the ΔluxR, exsB::cat strain containing the complementation plasmid at levels equivalent to those in the ΔluxR strain (data not shown). Therefore, exsB alone was unable to complement the exsB::cat mutation.

We reasoned that the failure of exsB to complement could be due to polar effects of the chromosomal exsB::cat mutation on downstream gene expression. To test this possibility, two more complementation constructs were engineered, one encoding exsB and the 601-bp intergenic region immediately downstream of the gene and one encoding exsB, this intergenic region, as well as the downstream gene exsA (Fig. 5A). Only the construct containing both exsB and exsA restored T3SS gene expression in the ΔluxR, exsB::cat mutant strain (Fig. 5A). Therefore, the chromosomal exsB::cat mutation appears to lead to a reduction in T3SS gene expression due to polar effects on exsA because exsB and exsA exist in an operon. The orientation of these two genes is reminiscent of the exsB and exsA genes of *Pseudomonas aeruginosa*, which have been shown to be expressed in the exsCEBA operon (6, 21, 44). Although the exsA gene of *V. harveyi* is 44% identical to its *P. aeruginosa* exsA homolog, the exsB genes of these organisms are only 13% identical. Moreover, *V. harveyi* does not appear to encode an exsE homolog, and the exsC gene is divergently transcribed from exsB (Fig. 1A).

To determine if the *V. harveyi* exsB and exsA genes are cotranscribed, RNA was harvested from a ΔluxR, exsB::cat mutant strain containing a vector control or three cloned regions from pCMW209 was measured in the ΔluxR and ΔluxR, exsB::cat mutant strains containing a vector control or three cloned regions carrying portions or all of the exsB4 operon as indicated. Error bars indicate standard deviations. (B) Agarose gel electrophoresis analysis of PCR amplification products using primers complementary to the 3′ end of exsB and the 5′ end of exsA to genomic DNA (lane 2), reverse transcriptase-negative control (lane 3), and cDNA generated from a ΔluxR mutant (lane 4). Lane 1 shows the DNA ladder.
ExsA is epistatic to QS in the control of T3SS gene expression. The relative expression of VIBHAR_01699 in the four indicated strains carrying a vector control (black) or overexpressing ExsA-FLAG (gray) was determined using quantitative PCR. Error bars indicate standard deviations.

ExsA is regulated by QS and is required for T3SS gene expression. ExsA, a member of the AraC/XylS family of transcriptional activators, activates the expression of T3SS genes in *P. aeruginosa* (18, 45), *Y. enterocolitica* (13), and *V. parahaemolyticus* (47). Our data suggest that QS regulates the production of the T3SS of *V. harveyi* through the control of exsA expression. To test this prediction, the expression of exsA was measured using quantitative PCR in the same two high-cell-density (wild-type and ΔluxO) and low-cell-density (ΔluxO ΔluxR) strains of *V. harveyi* used for the analysis of exsB expression (Fig. 3). Indeed, the expression pattern for exsA is similar to that for exsB. That is, like exsB, exsA is maximally expressed at low cell density and minimally expressed at high cell density.

In *P. aeruginosa*, *Y. enterocolitica*, and *V. parahaemolyticus*, exsA mutants are defective in T3SS gene expression (6, 7, 12, 40, 47). To determine if this is also the case for *V. harveyi*, we constructed an in-frame deletion of exsA in the ΔluxR background and measured the expression of VIBHAR_01699, the second gene in the T3SS.IV operon for transcription start sites using 5′-RACE analysis. Consistent with our results described above, VIBHAR_01699 was more highly expressed in the ΔluxR strain than in the wild-type strain, and mutation in exsB in the ΔluxR background reduced VIBHAR_01699 expression (Fig. 6, black bars). The deletion of exsA in the ΔluxR background also reduced VIBHAR_01699 expression to the levels observed for the wild-type strain. Thus, ExsA is required for the activation of T3SS gene expression in *V. harveyi*.

ExsA is the regulator connecting QS to T3SS gene expression. The data in Fig. 5A show that ExsA is necessary for the expression of T3SS genes in the ΔluxR, exsB::cat mutant strain. Is ExsA sufficient? To answer this question, the entire coding sequence of exsA containing a C-terminal FLAG tag was cloned under the control of the Ptac promoter and an exogenous translation start site on a complementation plasmid. This plasmid was introduced into four *V. harveyi* strains: the wild type (i.e., high cell density), ΔluxR (i.e., low cell density), ΔluxR, exsB::cat, and ΔluxR, ΔexsA, and T3SS gene expression was measured using quantitative PCR, again probing for VIBHAR_01699 in every strain examined, confirming that ExsA expression is sufficient to induce the expression of T3SS genes (Fig. 6, gray bars). Notably, induction occurs in the high-cell-density wild-type strain in which QS normally represses T3SS, indicating that the production of ExsA is epistatic to LuxR in controlling the expression of T3SS genes in *V. harveyi*.

The exsBA operon encodes two QS-regulated promoters. Taken together, the results described above suggest that QS, via LuxR, regulates the expression of the exsBA operon, and ExsA in turn controls T3SS gene expression. To determine if the regulation of exsBA by LuxR is direct, we analyzed the exsBA operon for transcription start sites using 5′-RACE analysis. One putative transcription start site was identified 370 bp upstream of the translation start site of exsA (Fig. 1A; termed P_{A} to denote exsA), and a second was identified 38 bp upstream of the translation start site of exsB (termed P_{B} to denote exsB; marked by arrows in Fig. 1A).

To determine if these putative transcription start sites contain functional promoters, we constructed luciferase transcriptional fusions to each potential promoter. The exsB-lux promoter (P_{B}) fusion encodes the DNA sequence 535 nucleotides upstream of the exsB translation start to 17 nucleotides downstream of the translation start site. The exsA-lux promoter (P_{A}) fusion encodes the entire intergenic region between exsB and exsA from 109 nucleotides upstream of the exsB stop codon to 125 nucleotides downstream of the exsA translation start site. Luciferase production was measured in a *V. harveyi* strain that is wild type for QS but contains a Tn5 insertion in the luxA gene, the ΔluxR mutant, and the ΔluxR, ΔexsA mutant. None of these three strains is bioluminescent, making them suitable hosts for the analysis of the lux transcriptional fusions. However, to ensure that the lux measured in this experiment was generated by the reporter plasmid and not the chromosomally encoded luciferase operon, luminescence from the promoterless parent vector, pBBRlux, also was determined (Fig. 7). Luciferase production from the exsB promoter fusion oc-
curred in the ΔluxR mutant and the ΔluxR, ΔexsA strains; however, expression in the luxA::Tn5 mutant strain was equal to the background bioluminescence from the vector control (Fig. 7). This result indicates that the PB promoter is functional and is repressed in the high-cell-density QS state. Furthermore, the expression of the exsB-lux promoter construct also was significantly reduced in the ΔluxR, ΔexsA mutant compared to that of the ΔluxR mutant (Fig. 7), suggesting that ExsA regulates its own expression through the autoactivation of the PB promoter driving the expression of the exsBA operon.

Bioluminescence expression above background levels also was observed in the exsA-lux promoter reporter, although overall expression from this fusion was 10-fold lower than that from the exsB-lux promoter construct (Fig. 7). The exsA-lux promoter was strongly repressed in the luxA::Tn5 mutant compared to expression in the ΔluxR strain; however, no significant difference in expression was observed between the ΔluxR and ΔluxR, ΔexsA strains. These results show that the PA promoter is functional, although it is not expressed as highly as the PB promoter. Like the PB promoter, the PA promoter is repressed by QS. However, unlike the PB promoter, the PA promoter is not regulated by ExsA.

**LuxR directly represses the PB promoter.** Two lines of evidence suggest that QS primarily controls exsA expression through the transcriptional regulation of the PB promoter. First, the PB promoter is expressed 10-fold more than the PA promoter at low cell density, but is repressed to low levels in the high-cell-density QS state. Second, the PA promoter is intact in the exsB::cat mutant, yet T3SS promoters are not expressed in this mutant, suggesting that insufficient ExsA is produced from the PA promoter to drive the transcription of the T3SS operons.

To examine the QS regulation of the PB promoter, we analyzed the surrounding nucleotide sequence for potential LuxR binding sites using a recently reported LuxR binding site prediction algorithm (33). Of note, no LuxR binding sites are predicted in the vicinity of the PA promoter. Two putative LuxR binding sites were identified near the PB promoter (Fig. 8A, underlined). One site is located at +5 to +22 relative to the transcription start site in the 5‘-untranslated region of the exsB4 mRNA, while the other site is located at the nucleotides encoded −43 to −63. We examined LuxR binding at these sites by performing EMSA with three regions of the exsBA promoter (Fig. 8B). The most upstream probe, containing a region of the exsBA promoter from −284→−116, does not possess a putative LuxR binding site, and, consistent with this, shows no interaction with LuxR (Fig. 8B, left). The central probe containing the region from −172→−6 that includes the predicted upstream LuxR binding site also did not bind to LuxR (Fig. 8B, middle). The most downstream probe, harboring the region from −55→+63 and encoding the second putative LuxR binding site, bound to LuxR (Fig. 8B, right). Therefore, we conclude that LuxR directly regulates the expression of exsBA through binding to a site located between the transcriptional and translational start sites of exsB (Fig. 8A).

**DISCUSSION**

In this study, we decipher the molecular mechanism by which QS controls the expression of T3SS genes in *V. harveyi*. At low cell density, when the concentration of AIs is low, LuxR is repressed, leading to the derepression of two promoters in the exsBA operon and the production of ExsA (and likely ExsB). ExsA, in turn, activates the expression of the operons encoding the T3SS structural genes. At high cell density, LuxR directly represses the transcription of the PB promoter located upstream of the exsBA operon, preventing the production of ExsA. Reduced levels of ExsA lead to the reduced expression of the four operons encoding the T3SS genes of *V. harveyi*.

QS regulation of T3SS has been reported for multiple bacteria. *P. aeruginosa*, like *V. harveyi* and *V. parahaemolyticus*, also represses T3SS at high cell density through a RhlR-C4-homoserine lactone autoinducer-dependent QS pathway (5). On the other hand, the expression of T3SS in enteropathogenic and enterohemorrhagic *E. coli* is reported to be induced at high cell density by the QS molecule AI-3 (36) through the
QseC/QseA two-component regulatory cascade (35, 37). It is unclear why different bacterial pathogens express T3SS at different cell densities, but presumably these distinct patterns optimize the particular disease process of each pathogen.

Although V. harveyi is not a human pathogen, V. harveyi does cause disease in marine organisms (1, 16, 46). The T3SS system of V. harveyi is proposed to be involved in these infections (24). The expression of T3SS genes at low cell density implies that only one or a few bacteria are sufficient to initiate disease by delivering effector proteins into target host cells. One can envision that, in the ocean, free-living V. harveyi cells exist at relatively low cell densities. Based on our results, under this condition, T3SS genes should be maximally expressed. Therefore, the T3SS appears poised to initiate infection upon an individual bacterium encountering a suitable host.

Using purified LuxR and EMSAs, we determined that LuxR directly regulates the exsBA operon in V. harveyi at the Pα promoter. This finding differs from that described for the QS regulation of the T3SS in P. aeruginosa, in which the operons encoding the structural genes for the T3SS are regulated by QS but the exsCEBA operon is not (5). LuxR acts as both an activator and a repressor; however, the parameters that govern when LuxR will activate gene expression and when LuxR will repress gene expression are not known. In the present work, we have determined that LuxR binds to a site located between the transcription start site and translation start site of exsB to repress expression. This promoter architecture resembles that described for hapR (encoding the LuxR homolog in Vibrio cholerae). In this case, HapR binding to its own promoter results in the autorepression of hapR expression (29). Thus, an emerging pattern is that the binding of LuxR-type proteins between the transcription and translation start sites of the target genes leads to repression rather than activation.

Our results identify two promoters located in the exsBA operon of V. harveyi that are regulated by QS, although it is unknown if the Pα promoter is directly controlled by LuxR. Two pieces of evidence suggest that the Pα promoter is the primary promoter controlling the transcription of exsBA. First, our studies indicated that expression from Pα is 10-fold higher than that from Pβ. In addition, mutations in exsB reduced the expression of exsA even though they are located hundreds of base pairs upstream of the Pβ promoter. This second result suggests that any ExsA produced from the Pβ promoter alone is not sufficient to induce the expression of the T3SS operons. Nevertheless, Pβ is clearly transcribed and QS regulated, suggesting that it plays some role in T3SS gene regulation through the modulation of exsA expression. Further experimentation is required to clarify this mechanism. Finally, similar to our results showing that the V. harveyi exsBA operon is autoactivated by ExsA, the P. aeruginosa exsCEBA operon also is activated by its ExsA homolog (6).

QS in V. harveyi controls numerous genes both positively and negatively. Of the approximately 60 promoters identified to be QS regulated to date, only 15 are known to directly interact with LuxR (24, 31, 33, 43). Thus, most of the genes in the LuxR regulon are indirectly controlled, suggesting that there exists a complex regulatory hierarchy emanating from LuxR to the control of the expression of downstream genes. Here, we describe one such multicomponent pathway that controls the expression of T3SS genes. In this case, LuxR functions as a repressor of the AraC-type transcriptional activator, ExsA, to promote T3SS expression exclusively at low cell density. In an analogous scenario, the LuxR repression of a repressor protein could promote the expression of target genes specifically at high cell density. As mentioned, LuxR also functions as a transcriptional activator. Having opposing regulatory abilities residing within one protein presumably provides V. harveyi with maximal flexibility to coordinate the expression of QS-controlled genes. Moreover, circuits involving additional regulatory layers could provide other mechanisms for the precise regulation of QS-controlled genes. These circuits likely function in a combinatorial fashion to direct the specific temporal expression of individual and collective behaviors at low and high cell density, respectively.

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