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* *exsBA* operon. While LuxR directly represses the expression of *exsBA*, 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 den-

sity (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 activities 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_01723 to VIBHAR_01742). The fourth structural operon, T3SS.4, is located approximately 15 kb away (VIBHAR_01692_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

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TABLE 1. Strain list

Strain	Description	Reference
BB120	Wild-type <i>V. harveyi</i>	2
KM83	<i>luxO(D47E)</i>	19
JW507	$\Delta luxR$	This study
MR100	$\Delta luxR$, <i>exsA::cat</i>	This study
MR100-flip	$\Delta luxR$, $\Delta exsA$	This study
JW510	$\Delta luxR$, <i>exsB::cat</i>	This study
JW510-flip	$\Delta luxR$, $\Delta exsB$	This study
JMH401	<i>luxO(D47E)</i> , <i>vopN::mini-MulacZ</i>	This study
KM470	<i>luxO(D47E)</i> , <i>vspC::mini-MulacZ</i>	This study
KM476	<i>luxO(D47E)</i> , <i>vopB::mini-MulacZ</i>	This study
JW103	$\Delta luxR$, <i>exsB::Tn5</i> , C6	This study
JW104	$\Delta luxR$, <i>exsB::Tn5</i> , E10	This study
BH421	<i>luxA::Tn5</i>	This study

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 $\Delta luxR$ 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. parahaemolyticus* 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 *exsBA* 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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MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. harveyi* strains were cultured in Luria-murine (LM) medium with shaking at 30°C. *Escherichia coli* S17- λ pir (15) was used for cloning procedures and was grown in Luria-Bertani medium at 37°C with shaking. Antibiotics were obtained from Sigma and used at the following concentrations (in μ g/ml): ampicillin, 100; kanamycin, 100; and tetracycline, 10. *V. harveyi* *exsB* and *exsA* mutants were constructed as previously described (39, 43). *V. harveyi* genomic DNA segments cloned into the cosmid pLAFR2 were mutated in *E. coli* using λ -red-mediated recombination (14). This process results in the complete deletion of the target gene from the start to stop codon and the insertion of the *cat* cassette from the plasmid pKD3. To generate unmarked deletions, FLP-mediated recombination was used, resulting in an 85-nucleotide (nt) scar (14). Mutations were transferred onto the *V. harveyi* chromosome using homologous recombination. Strains used in this study are listed in Table 1.

DNA manipulations. DNA manipulations were performed using standard procedures (34). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (NEB). PCRs were carried out with iProof DNA polymerase or Phire DNA polymerase (NEB). For cloning purposes, *exsB* and *exsA* were amplified by PCR from BB120 genomic DNA. The DNA for the complementation of the *exsB* mutant was amplified with primers containing BamHI

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 (17). *gfp* transcriptional fusions were constructed in pCMW1 (43) by cloning into the SphI and SalI 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 constructs were confirmed by sequencing. Primer sequences are available upon request.

Genetic screen to identify QS-controlled regulators of the T3SS. Tn5 mutagenesis of the *V. harveyi luxR* mutant JW507 was performed using the suicide vector pRL27c (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 (43), 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 sorted on a FACSaria flow cytometer (Becton Dickinson), and the lowest 1% of *gfp*-expressing transformants was isolated. The sorted cells were plated, and 4,000 colonies were individually transferred to 96-well plates (Costar 3595; Corning) containing 200 μ l LM medium with chloramphenicol. The plates were agitated for 20 to 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 and 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- λ pir by electroporation. Plasmids containing the transposon and flanking *V. harveyi* DNA were selected on LB medium with kanamycin and sequenced. Sequence analysis was performed using Vector NTI suite 10.1.1 (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, as this was the time point 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 μ l 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), RNA was isolated from *V. harveyi* strains grown to an optical density at 600 nm (OD_{600}) of 1.0 using the RNeasy mini kit (Qiagen). RNA was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Samples were treated with DNase I (Roche) in reaction mixtures containing 10 $\times$ PCR buffer (ABI) and 25 μ M MgCl₂ (ABI). cDNA was generated in 100- μ l reaction mixtures containing 1 μ g of RNA, 5 $\times$ first-strand buffer (Invitrogen), 100 μ M dithiothreitol (DTT) (Invitrogen), 10 μ M deoxynucleoside triphosphates (dNTPs) (ABI), random hexamers (Roche), and SuperScript II reverse transcriptase (Invitrogen). Reactions were completed 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 transcription enzyme. Real-time PCRs were performed on an ABI Prism 7900HT sequence detection system. Reactions were carried out in 384-well optical reaction plates (ABI) in quadruplicate with 2 $\times$ 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). *hfq*

mRNA was used as an endogenous loading control for the reactions. Relative mRNA levels using $\Delta\Delta CT$ 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 isomer] 5'-tagged fluorescent primers in a standard PCR and purified using the Zymoclean gel DNA recovery kit (Zymo Research). Each probe (10 nM, 5 μ l total volume) was combined with 1 μ l of 1- μ g/ml poly(dI-dC) and the indicated amount of LuxR (0 to 1,000 nM) in a final volume of 20 μ l at 30°C for 15 min. Thus, every reaction mixture contained a total of 14 μ l 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 *exsBA* and 5'-rapid amplification of cDNA ends (RACE) analysis. To determine if *exsB* and *exsA* are cotranscribed, RNA was prepared from a $\Delta luxR$ *V. harveyi* mutant grown to an OD₆₀₀ of 1.0 using the RNeasy protocol (Qiagen) and treated with Turbo DNase (Invitrogen). One μ g of RNA was converted to cDNA using iScript (Bio-Rad). A control reaction that did not contain reverse transcriptase but was otherwise identical also was performed. The primer set *exsAB-op-1A* (5'-GGCAGAGTGCATAAGGATTT-3'), located on the sense strand in the 3' end of the *exsB* genes, and *exsAB-op-3B* (5'-TAGTTGGCCTAACACATCCA-3'), located on the antisense strand in the 5' end of the *exsA* gene, was used to amplify 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 visualized by agarose gel electrophoresis.

For 5'-RACE analysis, RNA was prepared from *V. cholerae* wild-type (WT) cultures using the RNeasy protocol (Qiagen). The transcription start sites of *exsB* and *exsA* were determined using the 5'-RACE kit (Invitrogen) per the manufacturer's instructions. The primers used to map the transcription start site of *exsB* are VP1700-RACE-us-ds (5'-ACAAAAGAGTATTGCACAGG-3') and VP1700-GSP3 (5'-TTTGAGGAGTTGGTGTGAGT-3'), while primers used for *exsA* are VP1699-RACE-us-ds (5'-AGGAACACACCACAACCAGC-3') and VP1699-GSP3 (5'-ACGACAACAATGTGAGACTG-3').

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 $\Delta luxR$ 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 $\Delta luxR$ mutant and screened for mutants that showed the loss of the expression of pCMW209-cat.

Mutants containing random Tn5 insertions generated in a $\Delta luxR$ *V. harveyi* strain were pooled, pCMW209-cat was intro-

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 parahaemolyticus* 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, P_B and P_A , are indicated by arrows. (B) *exsD-gfp* expression from pCMW209-cat (bars 2 to 4) and from pCMW209 (bar 5) in the $\Delta luxR$ *V. harveyi* mutant and in three $\Delta luxR$, *exsB* mutants are shown. Error bars indicate standard deviations.

duced, and mutants exhibiting reduced *gfp* expression relative to that of pCMW209-cat in the parent $\Delta luxR$ 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 hydrolase; 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). 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 assessed in *trans* from the

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 $\Delta luxR$ *V. harveyi* (black bars) and in the $\Delta luxR, exsB::cat$ 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.

plasmid pCMW209-*cat*. Therefore, the reduction in *gfp* expression resulting from Tn5 insertions in *exsB* cannot be due to polar effects of the transposon on the plasmid-encoded T3SS.IV promoter. To further examine if *exsB* is part of the regulatory pathway connecting QS to the expression of T3SS genes, the chromosomal *exsB* gene in the *V. harveyi* $\Delta luxR$ mutant was replaced with the *cat* gene encoding resistance to chloramphenicol. The expression of *exsD-gfp* from pCMW209 in the $\Delta luxR, exsB::cat$ double mutant was reduced compared to that of the $\Delta luxR$ parent, confirming the phenotypes of the transposon mutants isolated in the genetic screen (Fig. 1B).

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 $\Delta luxR$ and $\Delta luxR, exsB::cat$ 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 $\Delta luxR, exsB::cat$ double mutant compared to that of the $\Delta luxR$ 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 $\Delta luxR, exsB::cat$

FIG. 3. QS represses *exsBA* expression. Quantitative PCR was used to determine the relative levels of *exsB* and *exsA* mRNA in four *V. harveyi* strains: wild type (white), $\Delta luxO$ (gray), *luxO(D47E)* (checkered), and $\Delta luxR$ (black). Error bars indicate standard deviations.

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 promoter 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 $\Delta luxO$ mutant that is locked into the high-cell-density state, and the *luxO(D47E)* and $\Delta luxR$ mutants that are locked into the low-cell-density state. The $\Delta luxO$ mutant is locked at high cell density because it does not express *qrr* genes, 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 $\Delta luxR$ 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 two low-cell-density-locked strains than in the wild-type and $\Delta luxO$ 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-

FIG. 4. Secretion is not required for the expression of T3SS genes in *V. harveyi*. *exsD-gfp* expression from pCMW209 was determined in the indicated strains. The white bar shows the background fluorescence of *V. harveyi*. Error bars indicate standard deviations.

erally, 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-MulacZ*), the T3SS.2 operon (*vopN::mini-MulacZ*), and the T3SS.3 operon (*vscP::mini-MulacZ*). 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 $\Delta luxR$, *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 $\Delta luxR$ strain and low in the $\Delta luxR$, *exsB::cat* strain. However, the introduction of the *exsB* gene alone did not restore the transcription of *exsD-gfp* in the

FIG. 5. *exsB* and *exsA* exist in an operon. (A) *exsD-gfp* expression from pCMW209 was measured in the $\Delta luxR$ and $\Delta luxR$, *exsB::cat* mutant strains containing a vector control or three cloned regions carrying portions or all of the *exsBA* 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 $\Delta luxR$ mutant (lane 4). Lane 1 shows the DNA ladder.

$\Delta luxR$, *exsB::cat* mutant (Fig. 5A). Using quantitative PCR, we confirmed that *exsB* mRNA was produced in the $\Delta luxR$, *exsB::cat* strain containing the complementation plasmid at levels equivalent to those in the $\Delta luxR$ 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 $\Delta luxR$, *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 $\Delta luxR$ mutant, treated with DNase, and converted into cDNA. As a control, an equivalent amount of RNA was prepared under the same conditions, except that no reverse transcriptase was added (RT⁻). PCR was performed on genomic DNA, the RT⁻ con-

FIG. 6. 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.

tol, and the cDNA using primers complementary to the 3' end of *exsB* and the 5' end of *exsA*. Amplification was observed from the genomic DNA and cDNA but not the RT– control, indicating that *exsB* and *exsA* are cotranscribed (Fig. 5B).

***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 $\Delta luxO$) and low-cell-density [*luxO*(D47E) and $\Delta luxR$] 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 $\Delta luxR$ background and measured the expression of VIBHAR_01699, the second gene in the T3SS.IV operon (Fig. 1A), using quantitative PCR. Consistent with our results described above, VIBHAR_01699 was more highly expressed in the $\Delta luxR$ strain than in the wild-type strain, and mutation in *exsB* in the $\Delta luxR$ background reduced VIBHAR_01699 expression (Fig. 6, black bars). The deletion of *exsA* in the $\Delta luxR$ 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 $\Delta luxR, exsB::cat$ 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

FIG. 7. *exsBA* region encodes two QS-regulated promoters. The RLUs (luminescence/OD₆₀₀) of the pBBRLux vector, *exsB-lux*, and *exsA-lux* promoter fusions were measured in a *luxA::Tn5* mutant (black), a $\Delta luxR$ mutant (gray bars), and a $\Delta luxR, \Delta exsA$ mutant (hatched bars). Error bars indicate standard deviations. RLUs indicate bioluminescence/OD₆₀₀.

type (i.e., high cell density), $\Delta luxR$ (i.e., low cell density), $\Delta luxR, exsB::cat$, and $\Delta luxR, \Delta exsA$, and T3SS gene expression was measured using quantitative PCR, again probing for VIBHAR_01699 levels. The exogenous production of ExsA increases the expression of 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 $\Delta luxR$ mutant, and the $\Delta luxR, \Delta exsA$ 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-

FIG. 8. LuxR directly represses the P_B promoter. (A) The sequence of the P_B promoter controlling the *exsBA* operon is shown. The identified transcription start site is boxed and indicated by a +1. The translation start codon (GTG) is shown by capital letters and by an arrow. The two predicted LuxR binding sites are depicted by solid lines under the sequences. (B) EMSA results are shown for three probes containing different regions of the *exsBA* promoter as indicated by the coordinates relative to the transcription start site. Each probe (10 nM) was incubated with 0, 42, 250, 500, or 1,000 nM LuxR as indicated underneath the gel.

curred in the $\Delta luxR$ mutant and the $\Delta luxR$, $\Delta exsA$ 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 P_B 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 $\Delta luxR$, $\Delta exsA$ mutant compared to that of the $\Delta luxR$ mutant (Fig. 7), suggesting that ExsA regulates its own expression through the autoactivation of the P_B 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 $\Delta luxR$ strain; however, no significant difference in expression was observed between the $\Delta luxR$ and $\Delta luxR$, $\Delta exsA$ strains. These results show that the P_A promoter is functional, although it is not expressed as highly as the P_B promoter. Like the P_B promoter, the P_A promoter is repressed by QS. However, unlike the P_B promoter, the P_A promoter is not regulated by ExsA.

LuxR directly represses the P_B promoter. Two lines of evidence suggest that QS primarily controls *exsA* expression through the transcriptional regulation of the P_B promoter. First, the P_B promoter is expressed 10-fold more than the P_A promoter at low cell density, but it is repressed to low levels in the high-cell-density QS state. Second, the P_A 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 P_A promoter to drive the transcription of the T3SS operons.

To examine the QS regulation of the P_B 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 P_A promoter. Two putative LuxR binding sites were identified near the P_B 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 *exsBA* 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 to -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 to -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 to +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 P_B 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. Thus, 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 P_B 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 P_A promoter is directly controlled by LuxR. Two pieces of evidence suggest that the P_B promoter is the primary promoter controlling the transcription of *exsBA*. First, our studies indicated that expression from P_B is 10-fold higher than that from P_A . In addition, mutations in *exsB* reduced the expression of *exsA* even though they are located hundreds of base pairs upstream of the P_A promoter. This second result suggests that any ExsA produced from the P_A promoter alone is not sufficient to induce the expression of the T3SS operons. Nevertheless, P_A 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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REFERENCES

- Alvarez, J. D., B. Austin, A. M. Alvarez, and M. Reyes. 1998. *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J. Fish Dis.* **21**:313–316.
- Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman. 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* **9**:773–786.
- Bassler, B. L., M. Wright, and M. R. Silverman. 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol. Microbiol.* **13**:273–286.
- Bassler, B. L., M. Wright, and M. R. Silverman. 1994. Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi*. *Mol. Microbiol.* **12**:403–412.
- Blevins, S., C. Soscia, P. Nogueira-Orlandi, A. Lazdunski, and A. Filloux. 2005. Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* **187**:3898–3902.
- Brutinel, E. D., C. A. Vakulskas, K. M. Brady, and T. L. Yahr. 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol. Microbiol.* **68**:657–671.
- Brutinel, E. D., C. A. Vakulskas, and T. L. Yahr. 2009. Functional domains of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* type III secretion system. *J. Bacteriol.* **191**:3811–3821.
- Brutinel, E. D., and T. L. Yahr. 2008. Control of gene expression by type III secretory activity. *Curr. Opin. Microbiol.* **11**:128–133.
- Burghout, P., F. Beckers, E. de Wit, R. van Bortel, G. R. Cornelis, J. Tommassen, and M. Koster. 2004. Role of the pilot protein YscW in the biogenesis of the YscC secretin in *Yersinia enterocolitica*. *J. Bacteriol.* **186**:5366–5375.
- Cao, J. G., and E. A. Meighen. 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J. Biol. Chem.* **264**:21670–21676.
- Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczar, B. L. Bassler, and F. M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**:545–549.
- Cornelis, G., C. Sluiter, C. L. de Rouvoit, and T. Michiels. 1989. Homology between virF, the transcriptional activator of the *Yersinia* virulence regulon, and AraC, the *Escherichia coli* arabinose operon regulator. *J. Bacteriol.* **171**:254–262.
- Cornelis, G., J. C. Vanoortegem, and C. Sluiter. 1987. Transcription of the yop regulon from *Y. enterocolitica* requires trans acting pYV and chromosomal genes. *Microb. Pathog.* **2**:367–379.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**:6640–6645.
- de Lorenzo, V., and K. N. Timmis. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235**:386–405.
- Diggles, B. K., G. A. Moss, J. Carson, and C. D. Anderson. 2000. Luminous vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*. *Dis. Aquat. Organ.* **43**:127–137.

17. Dunn, A. K., D. S. Millikan, D. M. Adin, J. L. Bose, and E. V. Stabb. 2006. New rfp- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and lux expression in situ. *Appl. Environ. Microbiol.* **72**:802–810.
18. Frank, D. W., and B. H. Iglewski. 1991. Cloning and sequence analysis of a trans-regulatory locus required for exoenzyme S synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **173**:6460–6468.
19. Freeman, J. A., and B. L. Bassler. 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Mol. Microbiol.* **31**:665–677.
20. Freeman, J. A., B. N. Lilley, and B. L. Bassler. 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Mol. Microbiol.* **35**:139–149.
21. Goranson, J., A. K. Hovey, and D. W. Frank. 1997. Functional analysis of exsC and exsB in regulation of exoenzyme S production by *Pseudomonas aeruginosa*. *J. Bacteriol.* **179**:1646–1654.
22. Hammer, B. K., and B. L. Bassler. 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol. Microbiol.* **50**:101–104.
23. Hammer, B. K., and B. L. Bassler. 2007. Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U. S. A.* **104**:11145–11149.
24. Henke, J. M., and B. L. Bassler. 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J. Bacteriol.* **186**:3794–3805.
25. Henke, J. M., and B. L. Bassler. 2004. Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J. Bacteriol.* **186**:6902–6914.
26. Larsen, R. A., M. M. Wilson, A. M. Guss, and W. W. Metcalf. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch. Microbiol.* **178**:193–201.
27. Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen, and B. L. Bassler. 2004. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**:69–82.
28. Lilley, B. N., and B. L. Bassler. 2000. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol. Microbiol.* **36**:940–954.
29. Lin, W., G. Kovackova, and K. Skorupski. 2005. Requirements for *Vibrio cholerae* HapR binding and transcriptional repression at the *hapR* promoter are distinct from those at the *aphA* promoter. *J. Bacteriol.* **187**:3013–3019.
30. Makino, K., K. Oshima, K. Kurokawa, K. Yokoyama, T. Uda, K. Tagomori, Y. Iijima, M. Najima, M. Nakano, A. Yamashita, Y. Kubota, S. Kimura, T. Yasunaga, T. Honda, H. Shinagawa, M. Hattori, and T. Iida. 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet* **361**:743–749.
31. Mok, K. C., N. S. Wingreen, and B. L. Bassler. 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J.* **22**:870–881.
32. Park, K. S., T. Ono, M. Rokuda, M. H. Jang, K. Okada, T. Iida, and T. Honda. 2004. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infect. Immun.* **72**:6659–6665.
33. Pompeani, A. J., J. J. Irgon, M. F. Berger, M. L. Bulyk, N. S. Wingreen, and B. L. Bassler. 2008. The *Vibrio harveyi* master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters. *Mol. Microbiol.* **70**:76–88.
34. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Sircili, M. P., M. Walters, L. R. Trabulsi, and V. Sperandio. 2004. Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. *Infect. Immun.* **72**:2329–2337.
36. Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **96**:15196–15201.
37. Sperandio, V., A. G. Torres, and J. B. Kaper. 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. *Mol. Microbiol.* **43**:809–821.
38. Swartzman, E., M. Silverman, and E. A. Meighen. 1992. The *luxR* gene product of *Vibrio harveyi* is a transcriptional activator of the *lux* promoter. *J. Bacteriol.* **174**:7490–7493.
39. Tu, K. C., and B. L. Bassler. 2007. Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev.* **21**:221–233.
40. Vakulskas, C. A., K. M. Brady, and T. L. Yahr. 2009. Mechanism of transcriptional activation by *Pseudomonas aeruginosa* ExsA. *J. Bacteriol.* **191**:6654–6664.
41. Viboud, G. I., and J. B. Bliska. 2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annu. Rev. Microbiol.* **59**:69–89.
42. Waters, C. M., and B. L. Bassler. 2005. Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* **21**:319–346.
43. Waters, C. M., and B. L. Bassler. 2006. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev.* **20**:2754–2767.
44. Yahr, T. L., and D. W. Frank. 1994. Transcriptional organization of the trans-regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **176**:3832–3838.
45. Yahr, T. L., A. K. Hovey, S. M. Kulich, and D. W. Frank. 1995. Transcriptional analysis of the *Pseudomonas aeruginosa* exoenzyme S structural gene. *J. Bacteriol.* **177**:1169–1178.
46. Zhang, X.-H., and B. Austin. 2000. Pathogenicity of *Vibrio harveyi* to salmonids. *J. Fish Dis.* **23**:93–102.
47. Zhou, X., D. H. Shah, M. E. Konkel, and D. R. Call. 2008. Type III secretion system 1 genes in *Vibrio parahaemolyticus* are positively regulated by ExsA and negatively regulated by ExsD. *Mol. Microbiol.* **69**:747–764.