Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*

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The production of virulence factors including cholera toxin and the toxin-coregulated pilus in the human pathogen *Vibrio cholerae* is strongly influenced by environmental conditions. The well-characterized ToxR signal transduction cascade is responsible for sensing and integrating the environmental information and controlling the virulence regulon. We show here that, in addition to the known components of the ToxR signaling circuit, quorum-sensing regulators are involved in regulation of *V. cholerae* virulence. We focused on the regulators LuxO and HapR because homologues of these two proteins control quorum sensing in the closely related luminous marine bacterium *Vibrio harveyi*. Using an infant mouse model, we found that a *luxO* mutant is severely defective in colonization of the small intestine. Gene arrays were used to profile transcription in the *V. cholerae* wild type and the *luxO* mutant. These studies revealed that the ToxR regulon is repressed in the *luxO* mutant, and that this effect is mediated by another negative regulator, HapR. We show that LuxO represses *hapR* expression early in log-phase growth, and constitutive expression of *hapR* blocks ToxR-regulon expression. Additionally, LuxO and HapR regulate a variety of other cellular processes including motility, protease production, and biofilm formation. Together these data suggest a role for quorum sensing in modulating expression of blocks of virulence genes in a reciprocal fashion *in vivo*.

The Gram-negative bacterium *Vibrio cholerae* usually inhabits natural aquatic environments, but it is best known as the causative agent of cholera, a severe diarrheal disease (1). Two factors are critical to *V. cholerae* virulence—cholera enterotoxin (CT) and an intestinal colonization factor known as the toxin-coregulated pilus (TCP). Poorly characterized environmental cues influence the expression of CT and TCP *in vivo* (2). Two sensory proteins, ToxR and TcpP, likely play a role in detection of the environmental signals, and then initiate a signal transduction cascade that promotes the expression of ToxT, which in turn, directly activates the transcription of genes involved in TCP and CT expression (3).

Recent work has established that many species of bacteria monitor their cell-population densities through the exchange of chemical signaling molecules (called autoinducers) that accumulate extracellularly and trigger alterations in behavior at high population densities. This phenomenon is referred to as quorum sensing (4, 5). Quorum sensing controls processes that include bioluminescence, virulence, biofilm formation, and sporulation in various bacterial species. In general, quorum sensing regulates processes that are effective only when a population of bacteria acts in a coordinated manner, but not when the bacteria act as individuals. Gram-negative bacteria typically use acyl-homoserine lactones as autoinducers (5, 6), whereas Gram-positive bacteria usually use modified oligopeptides as the communication signals (5–7). A link between quorum sensing and virulence has been established for only a few bacterial pathogens, most notably *Pseudomonas aeruginosa* (8) and *Staphylococcus aureus* (9).

The marine bacterium *Vibrio harveyi* uses a complicated quorum-sensing system to regulate bioluminescence and other phenotypes (10). *V. harveyi* produces two autoinducers, a homoserine lactone autoinducer (AI-1) and a second quorum-sensing autoinducer (AI-2) that was recently found to be a borate diester (11, 12). Detection of and response to AI-1 and AI-2 occurs through two parallel two-component signal transduction circuits (10, 13, 14), and a shared response regulator called LuxO integrates the information from these two circuits (15). LuxO negatively regulates luminescence expression, presumably by activating a putative downstream repressor of the luciferase operon (*luxCDABE*) (16). Additionally, a positive regulator called LuxR is required for transcription of the genes encoding luciferase (17, 18). LuxR is a close homologue of the *V. cholerae* protease regulatory protein HapR (19).

Analysis of the completed *V. cholerae* genome (20) reveals that although *V. cholerae* lacks the genes required for light production (*luxCDABE*), it possesses the genes required for production and response to the quorum-sensing signal AI-2 (*luxS, PQ, OU*). Consistent with this finding, earlier work demonstrated that *V. cholerae* produces AI-2 (21, 22). However, the *V. cholerae* target(s) controlled by this putative quorum-sensing circuit, if any, remained unknown. Here we show that the *V. harveyi*-like quorum-sensing regulators are involved in controlling *V. cholerae* virulence gene expression. Surprisingly, in contrast to other bacterial species in which quorum sensing activates virulence gene expression at high cell densities, in *V. cholerae*, quorum sensing appears to repress ToxR-regulated virulence genes.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. *V. cholerae* El Tor strain C6706 (23) was used as the parental strain in this study. The method used for construction of the *hapR* and *luxO* deletion mutants was that of Skorupski and Taylor (24). The p*hapR* plasmid was constructed by cloning the *hapR* ORF into pBBR1MCS4 (25) downstream of the constitutive p*lac* promoter. An isopropyl β-D-thiogalactoside (IPTG)-inducible *hapR*-expressing plasmid (pJZ146) was constructed by subcloning the *hapR* gene into pMal-c2x (New England Biolabs). pMal-c2x contains the p*tac* promoter and the *lacI*^{Q} gene. Chromosomal *tcpP-lacZ* and *hapR-lacZ* transcriptional reporter fusions were constructed by PCR amplifying the 5′ DNA of *tcpP* and *hapR*, respectively, and cloning these fragments into pVIK112 (26). The resulting plasmids were then integrated into the chromosome at the *tcpP* and *hapR* loci, respectively, by homologous recombination. When CT and TCP production was required, *V. cholerae* strains were grown in AKI medium as described (27). In all other experiments, *V. cholerae* strains were propagated in LB at 37°C.

Abbreviations: CT, cholera enterotoxin; TCP, toxin-coregulated pilus; IPTG, isopropyl β-D-thiogalactoside; HA, hemagglutinin.

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Microarray Experiments. The production of spotted microarrays containing full-length ORFs derived from *V. cholerae* strain N16961 has been described (28). RNA was isolated from cells grown in AKI medium by using TRIzol reagent (GIBCO/BRL), extracted with hot phenol (pH 5.2, 65°C), and purified by using a RNeasy kit (Qiagen, Chatsworth, CA). Fluorescently labeled cDNA was prepared by direct incorporation of fluorescent nucleotide analogues (Cy3-dCTP and Cy5-dCTP) during a first-strand randomly primed reverse transcription reaction. The differentially labeled cDNAs were combined and subsequently applied to the array surface under conditions that favor hybridization (28). Microarray slides were scanned by using a ScanArray 5000 apparatus (GSI Lumonics, Watertown, MA). For every ORF-specific spot, the resulting fluorescence intensity of each of the labels was measured and compared by using the GENEPIX PRO 3.0 software system (Axon Instruments, Foster City, CA).

Detection of CT, TCP, and Hemagglutinin (HA) Protease. GM1 ganglioside enzyme-linked immunosorbent CT assays (29) were performed after incubation of *V. cholerae* strains for 5 h at 37°C, with aeration in AKI medium. Cell extracts prepared from cultures used for CT assays were subjected to SDS/PAGE, transferred to nitrocellulose membranes, probed with anti-TcpA antibody (30), and visualized by using the enhanced chemiluminescence detection system (Amersham Pharmacia). HA protease azocasein assays were performed as described (31). Zymograms were performed by incorporating 0.2% (final) gelatin into 7% SDS/PAGE gels. Sample buffer was added to culture supernatants that subsequently were applied to the gels without boiling. After electrophoresis, gels were rinsed for 30 min in 2.5% and then 1% Triton X-100, followed by three rinses in protease buffer (0.1 M Tris, pH 8/0.5 mM $CaCl_2$). In-gel protease activity was allowed to proceed overnight in protease buffer at 37°C, after which gels were stained with Coomassie blue.

Biofilm Assays. Overnight cultures of *V. cholerae* were inoculated at a 1:100 dilution into LB broth and incubated in borosilicate tubes for 18 h at 22°C. Subsequently, the tubes were rinsed with distilled water then filled with crystal violet stain. After 5 min, the tubes were rinsed. The biofilm-associated crystal violet was resuspended with DMSO, and the OD_{570} of the resulting suspension was measured.

Infant Mouse Colonization Assay. The infant mouse colonization assay has been described (32). Briefly, *V. cholerae* mutant strains (Lac^+) were mixed with the wild-type strain (Lac^-), and approximately 10^5 cells were inoculated into 5- to 6-day-old CD-1 suckling mice. After a 20 h period of colonization, intestinal homogenates were collected, and the ratio of mutant/wild-type bacteria was determined by plating on LB agar containing 5-bromo-4-chloro-3-indolyl β-D-galactoside.

Results

LuxO Is Required for *V. cholerae* to Colonize Mice. The *V. harveyi luxCDABE* operon was used as a heterologous target in *V. cholerae* to test for quorum-sensing regulation. This study revealed that wild-type *V. cholerae* expresses luminescence in a cell density-dependent manner, a *luxO* mutant displays maximal constitutive luminescence, and a *hapR* mutant produces no light (M.B.M. and B.L.B., unpublished work). These *lux* expression patterns correspond exactly to those of the analogous *V. harveyi* strains, confirming that the *V. harveyi*-like quorum-sensing circuit is operational in *V. cholerae*, and also that the *V. cholerae* LuxO and HapR proteins are functional homologues of the *V. harveyi* LuxO and LuxR proteins, at least with respect to regulation of luciferase. However, *V. cholerae* does not possess a luciferase operon, so it is not clear what the endogenous quorum-sensing controlled targets are in this bacterium. The only previously characterized role for HapR is in regulation of expression of the HA protease (19).

Table 1. Infant mouse colonization assays

Strains	Competitive index
hapR	1.2
luxO	$<6.7 \times 10^{-4}$
luxOhapR	0.38

Assays were performed as described in *Materials and Methods.* The competitive index represents the ratio of output mutant to wild type (recovered from the intestine) divided by the ratio of input mutant to wild type (inoculated into the mouse). The *P* value for each independent experiment is <0.05 as determined by using the Student's *t* test.

In an effort to determine the targets of quorum sensing in *V. cholerae*, we tested whether mutations in *luxO* and/or *hapR* affect *V. cholerae* pathogenesis. To do this we performed an *in vivo* colonization assay using the infant mouse model. Six-day-old suckling CD-1 mice were infected with 1:1 mixtures of overnight-cultured wild-type and *luxO* mutants and wild-type and *hapR* mutants. Mice were killed after 20 h, and the small intestine was analyzed to determine the ratio of wild-type to mutant bacteria. Wild-type bacteria could be distinguished from the mutant strains by virtue of a *lacZ* deletion that does not affect virulence. Table 1 shows our results. The *hapR* mutant colonizes infant mice to the same extent as wild-type *V. cholerae* (competitive index ≈ 1). In contrast, the *luxO* mutant is profoundly defective in colonization, as not a single *luxO* mutant bacterium was recovered from the small intestines of any of the 12 mice used in the experiment. Therefore the competitive index for the *luxO* mutant is $<10^{-4}$.

LuxO Regulates Virulence Gene Expression. To investigate the mechanism by which LuxO regulates *V. cholerae* pathogenicity, we performed whole-genome DNA microarray experiments and compared the transcription profiles of the wild-type and *luxO* mutants. In this experiment we used *in vitro* conditions (AKI medium) that are known to induce the expression of virulence factors. Our results are presented in Fig. 1 and Table 2. Fig. 1 shows that, relative to the wild-type strain, many genes are activated and many are repressed in the *luxO* mutant. However, higher expression of all of the genes in the ToxR-virulence regulon occurs in the wild-type strain com-

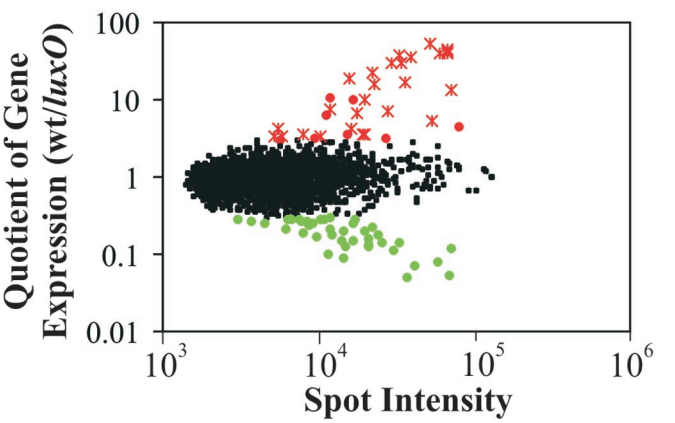

Fig. 1. Gene expression profiles for wild-type C6706 and the *luxO V. cholerae* mutant. Genes with higher expression in the wild-type strain compared with the *luxO* mutant are shown in red. Genes expressed at higher levels in the *luxO* mutant compared with the wild-type strain are shown in green. Black dots represent genes that displayed less than 3-fold variation between the two strains. * indicate genes known to be essential for virulence.

Table 2. Differential gene expression in the *luxO* mutant in AKI medium

ORF	ID	Fold activation
Pathogenesis		
ToxR-activated gene A protein, TagA	VC0820	−3.6
TagD protein	VC0824	−13.4
Toxin coregulated pilus biosynthesis protein, TcpI	VC0825	−7.5
Toxin coregulated pilus biosynthesis protein, TcpP	VC0826	−16.6
Toxin coregulated pilus biosynthesis protein, TcpH	VC0827	−10.2
Toxin coregulated pilin, TcpA	VC0828	−41.7
Toxin coregulated pilus biosynthesis protein, TcpB	VC0829	−29.3
Toxin coregulated pilus biosynthesis protein, TcpQ	VC0830	−22.1
Toxin coregulated pilus biosynthesis protein, TcpC	VC0831	−35.9
Toxin coregulated pilus biosynthesis protein, TcpR	VC0832	−18.8
Toxin coregulated pilus biosynthesis protein, TcpD	VC0833	−37.8
Toxin coregulated pilus biosynthesis protein, TcpS	VC0834	−53.9
Toxin coregulated pilus biosynthesis protein, TcpT	VC0835	−29.7
Toxin coregulated pilus biosynthesis protein, TcpE	VC0836	−26.9
Toxin coregulated pilus biosynthesis protein, TcpF	VC0837	−45.0
TCP pilus virulence regulatory protein, ToxT	VC0838	−2.7
Leader peptidase, TcpJ	VC0839	−18.0
Accessory colonization factor, AcfB	VC0840	−19.7
Accessory colonization factor, AcfC	VC0841	−16.3
TagE protein	VC0843	−10.5
Accessory colonization factor, AcfA	VC0844	−28.4
Cholera enterotoxin, B subunit, CtxB	VC1456	−40.4
Cholera enterotoxin, A subunit, CtxA	VC1457	−39.5
Haemolysin, HlyA	VCA0219	−6.9
Membrane proteins or secretion		
Transporter, NadC family	VCA0025	−6.3
Outer membrane protein, OmpV	VC1318	−4.5
Iron(III) ABC transporter	VCA0687	−3.6
Amino acid ABC transporter	VC1362	4.9
Porin, OmpT	VC1854	5.5
Metabolism		
Aldehyde dehydrogenase, AldA-1	VC0819	−12.7
Glycerol kinase, GplK	VCA0744	−5.3
Hydrolase	VCA0877	6.9
Acetyl-CoA acetyltransferase	VCA0690	8.1
Polyhydroxyalkanoic acid synthase, PhaC	VCA0688	9.1
Acetoacetyl-CoA reductase	VCA0691	9.9
Chemotaxis and motility		
Methyl-accepting chemotaxis protein	VC1248	6.3
Purine-binding chemotaxis protein, CheW	VC1402	5.5
Methyl-accepting chemotaxis protein	VC1403	4.9
Methyl-accepting chemotaxis protein	VCA0031	3.4
Chemotactic transducer-related protein	VCA0895	4.0
Methyl-accepting chemotaxis protein	VCA0979	4.2
Methyl-accepting chemotaxis protein	VCA1092	4.8
Chemotaxis protein, CheA	VCA1095	3.8
Chemotaxis protein, CheY	VCA1096	3.7
Regulation		
Sensory box sensor histidine kinase	VCA0211	3.6
Hemagglutinin/protease regulatory protein, HapR	VC0583	6.9
Unknown functions		
Enterobactin synthetase	VC1579	−9.9
Hcp protein	VC1415	4.8
Hcp protein	VCA0017	7.1

Positive values represent activation in *luxO* mutants, negative values represent repression. Expression of 20 other conserved hypothetical genes and hypothetical genes is changed greater than 3-fold in *luxO* mutants (data not shown).

pared with the *luxO* mutant. The differences in expression level for all of the LuxO-regulated genes are given in Table 2. Again, these data clearly show that the entire ToxR regulon is repressed in the *luxO* mutant. For example, genes in the TCP island are repressed 2.7- to 41.7-fold in the *luxO* mutant. Similarly, the *ctxA* and *ctxB* genes, which are transcribed in an operon and encode the two subunits of CT, are also strongly inhibited (40-fold) in the *luxO* mutant.

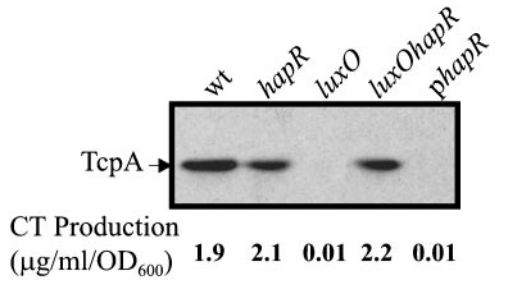

Fig. 2. CT and TcpA production in wild-type C6706 and mutant *V. cholerae* strains. Samples were prepared after 5 h incubation in AKI medium with aeration at 37°C. (*Upper*) Cell pellets from the specified strains were subjected to Western blot and probed with anti-TcpA antibody. (*Lower*) The corresponding cell-free culture fluids were assayed in GM1 ganglioside enzyme-linked immunosorbent CT assays.

To verify the microarray data, we examined the production of two major virulence determinants, CT and TcpA, after growth in AKI medium under exactly the same conditions we used in the microarray experiment. Our results show that the *luxO* mutant does not produce any detectable TcpA (Fig. 2 *Upper*) or CT (Fig. 2 *Lower*). These data are consistent with the microarray experiment and explain why the *luxO* mutant fails to colonize mice.

LuxO Acts at the Level of *tcpP* Expression. Two parallel signal transduction cascades coordinately regulate virulence in *V. cholerae* (33). The ToxR–ToxS and the TcpP–TcpH signaling circuits detect and respond to environmental stimuli, and both circuits exert their regulatory control over virulence by influencing the expression of *toxT*. ToxT, in turn, activates the transcription of a variety of genes required for virulence (34). To study the mechanism of repression of the virulence regulon in the *luxO* mutant, we constitutively expressed ToxR, TcpP, and ToxT (33) in the *luxO* mutant and tested whether overexpression of any of these genes could bypass the *luxO* defect and restore the mutant to CT production. Fig. 3*A* shows that constitutive expression of *toxR* does not affect CT production in the *luxO* mutant. However, CT is produced when either *tcpP* or *toxT* is constitutively expressed in the *luxO* mutant. This result indicates that the LuxO effect on the virulence regulon is mediated through down-regulation of TcpP. Consistent with this hypothesis, the microarray data in Table 2 show that 16.6-fold lower

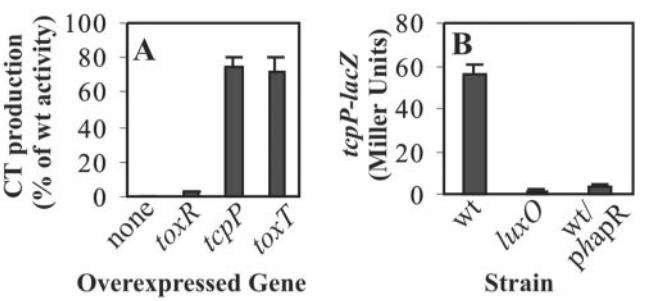

Fig. 3. LuxO represses *tcpP* expression. (*A*) *toxR*, *tcpP*, and *toxT* were cloned into the expression vector pBAD24 (33), and the plasmids were introduced into the *V. cholerae luxO* mutant. Subsequently, the strains were grown under AKI-inducing conditions in the presence of 0.01% arabinose. CT production was quantitated after 5 h incubation at 37°C with aeration. The data are presented as the percentage of CT production of the wild-type bearing the same plasmids. (*B*) *tcpP-lacZ* expression was assayed in the wild type, the *luxO* mutant, and the wild-type strain constitutively expressing a cloned *hapR* gene (denoted p*hapR*). β-galactosidase activity assays (39) were conducted after growth with aeration for 5 h at 37°C in AKI medium.

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expression of *tcpP* occurs in the *luxO* mutant than in the wild-type strain.

In an independent test to verify that LuxO activates *tcpP* expression, we introduced *tcpP-lacZ* transcriptional reporter fusions onto the chromosomes of the wild-type and *luxO* null mutant. The β-galactosidase activity of the *tcpP-lacZ* fusion was measured in each strain, and we found that 40-fold less expression of the reporter occurs in the *luxO* mutant than in the wild type (Fig. 3*B*). This result shows that transcription of *tcpP* requires the presence of a functional LuxO protein.

LuxO Acts Through HapR to Control *tcpP* Expression. The microarray experiment reveals that one of the genes regulated by LuxO is the *V. harveyi luxR* homologue *hapR*. Specifically, *hapR* expression increases 6.9-fold in the *luxO* mutant, suggesting that LuxO is a repressor of *hapR* expression (Table 2). We therefore wondered whether LuxO might affect *tcpP* expression indirectly, by acting through HapR. We hypothesized that LuxO negatively regulates *hapR* expression, and HapR in turn represses *tcpP* expression. To test this idea, a plasmid containing a constitutively expressed *hapR* gene was introduced into wild-type *V. cholerae*. The resulting recombinant strain fails to produce TCP and CT under our standard inducing conditions (Fig. 2), indicating that HapR is involved in repression of the ToxR regulon. Consistent with this result, Fig. 3*B* shows that in a wild-type strain, constitutive expression of *hapR* results in nearly complete repression of the *tcpP-lacZ* reporter fusion. Microarray analysis also demonstrates that constitutive expression of *hapR* results in a transcriptional profile of *V. cholerae* virulence genes very similar to that of the *luxO* mutant (data not shown). Taken together, these data suggest that HapR acts downstream of LuxO to repress *tcpP* expression, and this action ultimately results in repression of the ToxR virulence regulon.

To confirm our predictions regarding the roles of LuxO and HapR in virulence gene regulation, we performed an epistasis test to show that HapR indeed acts downstream of LuxO. We constructed a *luxO*, *hapR* double null mutant and tested CT and TCP production as well as the ability of the double mutant to colonize mice. The double mutant produces wild-type levels of both CT and TCP (Fig. 2), and this mutant has, at most, only a minor colonization defect (Table 1). These data show that *hapR* is epistatic to *luxO* and that LuxO is therefore not required to directly activate the *tcpP* promoter. Rather, LuxO controls *tcpP* expression indirectly by repressing expression of *hap*R, which in turn represses *tcpP* expression. We do not know whether HapR acts directly or indirectly to repress *tcpP* transcription. In general, HapR and its homologues in other *Vibrio* species (LuxR, SmcR, and OpaR) act as transcriptional activators (17, 18, 36, 37). As mentioned previously, HapR activates the expression of *luxCDABE* in *V. cholerae*. Many activators are also repressors, however, and HapR could possess both activities. Alternatively, HapR could activate a downstream repressor of *tcpP* expression.

LuxO Regulation of *hapR* Expression. Our data suggest that HapR plays an important role in the quorum-sensing regulation of *V. cholerae* virulence factors. To further investigate how HapR functions in regulation of the virulence process, we monitored the expression of a *hapR-lacZ* transcriptional fusion in the wild-type and the *luxO* mutant *V. cholerae* strains as a function of cell density. Fig. 4*A* shows that *hapR* is expressed at low cell densities in the *luxO* null mutant, but not at low cell densities in wild-type *V. cholerae*. However, by the time the strains reach late log-phase, expression of *hapR* is identical in the wild-type and the *luxO* mutant. We interpret this result to imply that initial but not late expression of *hapR* in the *luxO* mutant is critical for the inhibition of virulence factor production.

A model in which early but not late expression of *hapR* represses virulence gene expression predicts that induction of

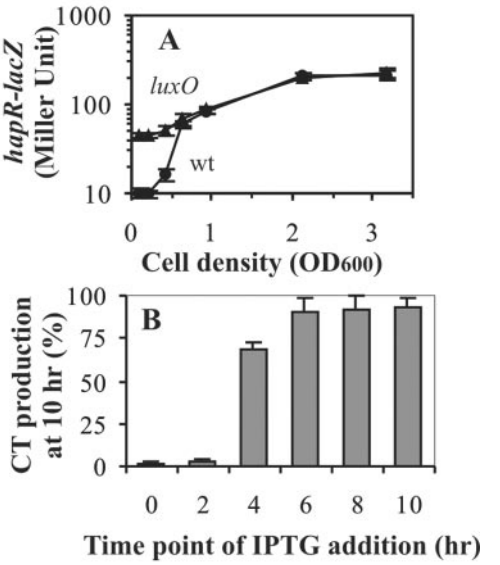

Fig. 4. LuxO regulation of *hapR* expression and HapR regulation of CT production. (*A*) Wild-type and *luxO* mutants carrying a chromosomal *hapR-lacZ* reporter fusion were grown in AKI medium at 37°C. Samples were withdrawn at the specified ODs, and β-galactosidase activity was assayed. ●, wild type (wt); ▲, *luxO* mutant. (*B*) Wild-type *V. cholerae* containing the vector pMal-c2x or carrying pJZ146 (pMal-c2X containing *hapR* under IPTG control) were grown as described in AKI medium, and IPTG (50 μM final concentration) was added at the indicated time points. All of the samples were assayed for CT production after a total of 10 h at 37°C. The data are presented as the percentage of CT production in the *V. cholerae* strain carrying pJZ146 compared with the strain carrying only the vector pMal-c2x.

hapR at late times should not influence the production of virulence factors. To test this idea, we introduced a plasmid containing an IPTG-inducible *hapR* gene construction into wild-type *V. cholerae* C6706. IPTG was added at different time points during growth, and CT production was assayed after 10 h of incubation. Fig. 4*B* shows the level of CT production in HapR-expressing cells compared with that produced by a strain containing a vector control. Fig. 4*B* shows that the HapR inhibitory effect on CT production is observed only when *hapR* is induced at early time points, even though SDS/PAGE analysis showed that the total HapR protein made by 10 h was similar regardless of whether IPTG was added at 0 h or 8 h (data not shown). These data indicate that, at least *in vitro*, HapR acts at an early stage of growth to repress virulence factor production. It remains unclear how HapR regulates virulence gene expression *in vivo*.

LuxO and HapR Proteins Control Multiple Processes in *V. cholerae*. We investigated whether LuxO and HapR might control multiple cellular processes in addition to pathogenesis. As mentioned, HapR is required for production of the HA protease (19). The HA protease is encoded by the *hapA* gene, and it is a major extracellular protease that may serve as a "detachase" during colonization (38). Zymogram analysis (Fig. 5*A*) of 17 h cell-free spent culture fluids from the *V. cholerae hapR* and *hapA* mutants shows that, as expected, these mutants produce no HA protease. Quantitative analysis of the protease activities of the *hapR* mutant shows that it is very similar to that of the *hapA* mutant (Fig. 5). In contrast, identical preparations made from the *luxO* mutant resulted in a zymogram and protease activity profile similar to that of the wild type (Fig. 5). Interestingly, our analyses demonstrate that higher levels of protease are present in cell-free spent culture fluids prepared from a *luxO-hapA* double mutant than from the *hapA* single mutant (Fig. 5*B*). This result indicates

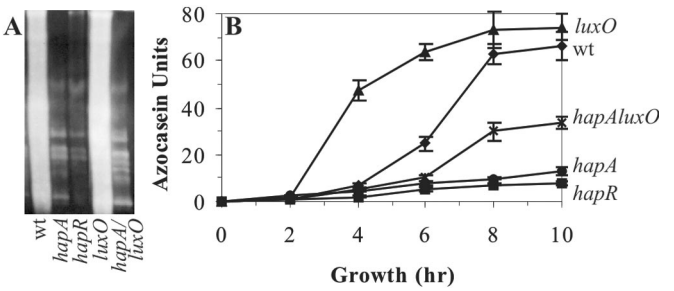

Fig. 5. HA protease production in *V. cholerae* wild-type and mutant strains. (*A*) Zymogram analyses of cell-free culture fluids prepared from the designated *V. cholerae* parent and isogenic mutant strains after 17 h incubation at 37°C are shown. In lanes prepared from samples containing high HA protease activity [wild type (wt) and *luxO*], the HA protease results in complete clearing of the gelatin because the HA protease is active during the electrophoresis run. (*B*) A time course of HA protease production is shown for the same strains analyzed in *A*. Overnight cultures were diluted 1:100 in LB and incubated at 37°C. Samples were taken at 2-h intervals for determination of azocasein activity. One azocasein unit is defined as the amount of enzyme producing an increase of 0.01 OD units per h.

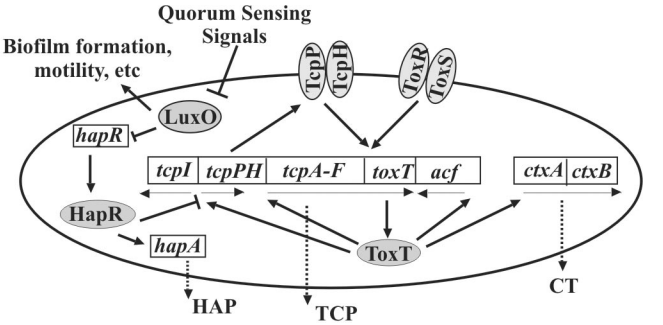

Fig. 7. A model for quorum-sensing regulation of *V. cholerae* virulence. Solid arrows denote positive effects while solid T bars denote negative effects. At low cell density, LuxO is active and represses the expression of *hapR*. HapR is a negative regulator of *tcpP* transcription, so under this condition, the TcpP signaling protein is present and it, together with TcpH and ToxRS, activates the expression of virulence factors TCP and CT. In contrast, at high cell density, LuxO is inactive as a result of autoinducer signal accumulation. Inactivation of LuxO results in *hapR* expression at high cell density. HapR represses TcpP and the ToxR regulon and activates Hap protease expression.

that LuxO negatively regulates secreted proteases other than the HA protease. Additionally, the time course of protease production (Fig. 5*B*) shows that protease activity is produced earlier in the *luxO* mutant than in the wild type. We suggest that this LuxO regulation is exerted through HapR, which is consistent with our finding that LuxO represses *hapR* expression only at low cell density.

The microarray data presented in Table 2 show that the expression of several genes involved in chemotaxis and motility are altered in the *luxO* mutant. We examined the motility of the *luxO* mutant on a swarm plate and found that this mutant is less motile than the wild-type and *hapR* mutant strains (Fig. 6*A*). We also tested whether LuxO and HapR are involved in biofilm formation. Photographs and crystal violet quantifications of biofilms produced by the wild-type, *luxO*, and *hapR* mutants are shown in Fig. 6*B*. Compared with wild-type *V. cholerae*, the *luxO* mutant is deficient in its ability to form a biofilm, whereas the *hapR* mutant is increased in its ability to form a biofilm.

Discussion

Bacteria coordinately control gene expression to adapt to and survive in fluctuating environmental conditions. For example, the human pathogen *V. cholerae* possesses a virulence regulon of more than 20 genes involved in colonization, toxin production, and bacterial survival within the host (33, 34). The virulence regulon is under the control of a cascade of transcriptional regulators that includes ToxR, TcpP, and ToxT. These regulators are hypothesized to respond to external cues such as temperature, pH, and osmolarity. In this study, we demonstrate a central role for the quorum-sensing proteins LuxO and HapR in the regulation of virulence gene expression in *V. cholerae*. The data presented here suggest that LuxO negatively regulates the expression of HapR, which in turn represses the expression of the essential virulence gene regulator, TcpP.

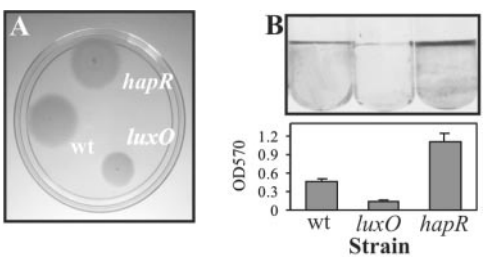

Fig. 6. LuxO and HapR control multiple processes in *V. cholerae*. (*A*) Different *V. cholerae* strains were inoculated into motility agar (LB containing 0.3% agar) and incubated at 37°C for 4 h, after which the photograph was taken. (*B*) A comparison of biofilms produced by wild-type *V. cholerae* and the *luxO* and *hapR* mutants. (*Upper*) The photograph shows the crystal violet staining in the borosilicate tubes containing the different strains. The normalized data are presented for these assays (*Lower*). The OD_{570} values are a measure of crystal-violet staining, which is proportional to the level of biofilm formation. The designation wt refers to the wild-type strain.

It remains unclear how quorum signals and other environmental cues are integrated to regulate virulence *in vivo*. One speculative model (Fig. 7) proposes that on initial (low bacterial cell density) colonization of a host by *V. cholerae* LuxO represses *hapR* and allows the expression of *tcpP.* This, in turn, results in the expression of the virulence factors in the ToxR regulon. These virulence factors enable *V. cholerae* to colonize the small intestine, multiply, and produce cholera toxin. When a high cell density is reached, autoinducer accumulates, and LuxO no longer represses *hapR* expression. Subsequent production of HapR presumably represses *tcpP* and ToxR regulon expression. In contrast, at high cell density, HapR activates the expression of *hapA*, which encodes the HA protease. Protease expression might promote detachment of *V. cholerae* cells, and thus facilitate establishment of new infection foci elsewhere within the gastrointestinal tract, or alternatively, promote the exit of *V. cholerae* from the host. Repression of the TCP, a type IV pilus proposed to mediate bacterial-bacterial adhesion, also might promote detachment of *V. cholerae* from the epithelium. It should be noted that, although we have demonstrated an unequivocal role for LuxO and HapR in virulence gene expression, our studies have not yet shown that virulence genes are responsive to autoinducers that accumulate at high cell density.

Interestingly, several toxigenic *V. cholerae* strains (e.g., El Tor strain N16961 and classical strain O395) possess a natural frame-shift mutation in the *hapR* gene (data not shown). These strains express low levels of HA protease, and a mutation in *luxO* does not result in a CT production defect (data not shown and ref. 39). This phenotype is similar to what we show here for the *luxO-hapR* double mutant. It is important to note that negative regulation of the *hapR* homologue *luxR* by LuxO in *V. harveyi* has not been observed. Therefore, this variation in the regulatory circuit could be unique to *V. cholerae*. We speculate that elimination of HapR, either by mutation or LuxO repression, may be an evolutionary step that improves *V. cholerae* adaptation

to the human host. Presumably, loss or down-regulation of HapR is advantageous for survival in and/or colonization of the host by prolonging TCP and CT expression even at high cell density. It is also likely that loss of HapR results in low HA protease production, which in turn could restrict detachment of bacteria from the intestinal epithelium. Thus, vibrios lacking HapR might remain attached to the epithelium for longer times. This could prolong colonization and the duration of shedding of vibrios in the stools of cholera sufferers. Also, as we have demonstrated, *hapR* mutants form thicker biofilms, and this may aid in their persistence within the host. Further studies should address how quorum-sensing autoinducers and other signals are coordinated to regulate the broad array of virulence-associated phenotypes of *V. cholerae*.

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