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A Constitutively Active Variant of the Quorum-Sensing Regulator LuxO Affects Protease Production and Biofilm Formation in *Vibrio cholerae*

Russell E. Vance, Jun Zhu, and John J. Mekalanos*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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Vibrio cholerae **normally inhabits aquatic habitats but can cause a severe diarrheal illness in humans. Its arsenal of virulence factors includes a secreted hemagglutinin (HA) protease. An HA protease-deficient mutant of *V. cholerae* was isolated and designated E7946 *mpc*. E7946 *mpc* was found to contain a point mutation in the *luxO* quorum-sensing regulator. In accordance with this finding, E7946 *mpc* exhibits a defect in quorum sensing. The mutant *luxO* allele [*luxO*(Con)] produces a protein with a leucine-to-glutamine substitution at amino acid 104. Transfer of the *luxO*(Con) allele to an otherwise wild-type background was sufficient to eliminate HA protease expression; conversely, deletion of *luxO*(Con) from E7946 *mpc* restored protease activity. We demonstrate that LuxO(Con) constitutively represses the transcription of *hapR*, an essential positive regulator of HA protease. Interestingly, strains harboring *luxO*(Con) form enhanced biofilms, and enhanced biofilm formation does not appear to be dependent on reduced HA protease expression. Taken together, the results confirm the role of LuxO as a central "switch" that coordinately regulates virulence-related phenotypes such as protease production and biofilm formation.**

Toxigenic *Vibrio cholerae* is the infectious agent of cholera, a severe diarrheal disease with 293,113 reported cases and 10,586 deaths worldwide in a typical year (1998), mostly in developing countries (28). The primary virulence factor of toxigenic *V. cholerae* is cholera toxin (CT), encoded by the *ctxAB* operon, which resides on a filamentous phage, CTXΦ (26). The toxin-coregulated pilus (TCP) has also been shown to be an essential colonization factor (12). *V. cholerae* strains lacking functional CT have been engineered for use as vaccines; however, some of these strains still exhibit residual reactogenicity in volunteers (25), suggesting that *V. cholerae* harbors toxic factors in addition to CT. In vitro studies (19, 29) have suggested that the secreted hemagglutinin (HA) protease of *V. cholerae*, encoded by the *hapA* gene (11), might be one such factor, as might a hemolysin, HlyA (23), and the recently described RTX toxin (8, 10, 18).

The environmental signals that promote expression of CT, HA protease, and other virulence factors remain largely undefined. Two membrane-localized complexes (TcpP/H and ToxR/S) have been shown to promote the transcription of *toxT*, a potent activator of CT and TCP transcription (5). Transcription of *tcpP/H* is itself activated by the synergistic action of AphA and AphB (24).

Recent work has also established that the LuxO protein, a σ^{54} dependent activator (15), is required for CT production in vitro and for colonization in an in vivo infant-mouse model (21, 30). LuxO was first characterized in *Vibrio harveyi* (1), where it has been shown to play a crucial role in "quorum sensing," the ability of bacteria to regulate gene expression in response to changes in cell density. LuxO in *V. cholerae* is thought to be phosphorylated and active at low cell density, where it represses (directly or indirectly) the transcription of *hapR* (30) (Fig. 1). At high cell density, LuxO is dephosphorylated and inactive and *hapR* is transcribed. HapR is an essential activator of the *hapA* protease gene (14) and also downregulates the expression of *tcpPH.* Recent evidence suggests that HapR decreases *tcpPH* transcription indirectly, by repressing the transcription of *aphA* (17).

Previously, a protease-defective mutant of *V. cholerae* El Tor strain E7946 was generated by Tn*5* mutagenesis (3, 19). The protease mutant was designated E7946 *mpc* (for "multiple protease control") since preliminary experiments suggested that *mpc* was defective in the secretion of multiple proteases (3). Here we report that *mpc* harbors a point mutation in *luxO* that constitutively activates LuxO function, represses protease production, and enhances biofilm formation by *V. cholerae.*

MATERIALS AND METHODS

Strains and plasmids. Streptomycin-resistant isolates of *V. cholerae* El Tor strains E7946, C6706, and N16961 were used. For selection of *V. cholerae*, antibiotics were used at the following concentrations: streptomycin at 100 μg/ml, tetracycline at 1.5 μg/ml, kanamycin at 50 μg/ml, and ampicillin at 100 μg/ml. For selection of *Escherichia coli*, tetracycline was used at 10 μg/ml and ampicillin was used at 100 μg/ml. *V. cholerae* and *E. coli* were grown in Luria-Bertani (LB) medium at 37 or 30°C as indicated. E7946 *mpc* was isolated from a Tn*5* mutagenesis experiment (3) and has been partially characterized (19). C6706 Δ*luxO* and C6706 Δ*hapR* have been described previously (30). The plasmid used to generate an in-frame deletion of *luxO* in E7956 *mpc* was the gift of M. Miller and B. Bassler. N16961 Δ*hapA* was the gift of K. Fullner.

A construct to transfer the point mutation in *luxO* from E7946 *mpc* into E7946 and C6706 wild-type strains was generated by amplifying a 2,017-bp segment of *luxO* (containing the point mutation) from E7946 *mpc* by using primers 5′1414Sac (ATAGAGCTCGGCTTACCAAGTGTTGACTCC) and 3′3431Xho (ATACTCGAGTTCTTCACCGATCTCCTTCG). The PCR product was digested with *Sac*I and *Xho*I and cloned into pKEK229 (4), a derivative of pCVD442 (6) that has the polylinker of pWSK30 (27). The resulting vector was transferred by homologous recombination onto the E7946 and C6706 chromosome by mating and selection for ampicillin and streptomycin resistance. Vector

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Armenise Bldg., Rm. 421, Boston, MA 02115. Phone: (617) 642-1935. Fax: (617) 738-7664. E-mail. jmekalanos@hms.harvard.edu.

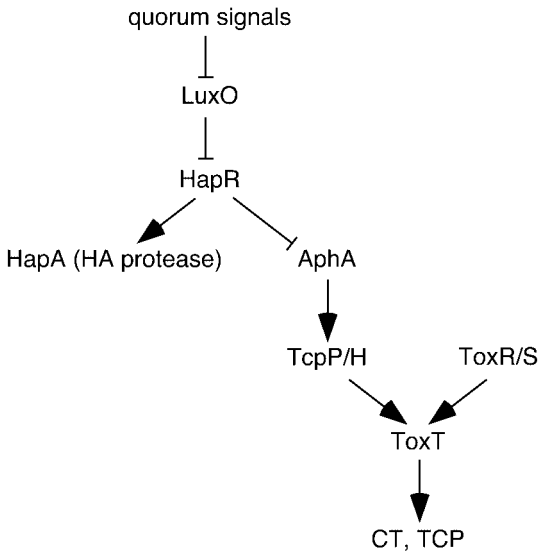

FIG. 1. Simplified scheme of quorum-sensing signaling in *V. cholerae.* During bacterial growth, cell-permeable chemical signals (autoinducers) are produced and accumulate to threshold levels at high cell densities. These signals lead to the dephosphorylation of LuxO, rendering LuxO inactive. At low cell densities, LuxO is phosphorylated and inhibits *hapR* transcription by an unknown mechanism (possibly indirectly, through stimulating the production of a *hapR* transcriptional repressor). HapR is required for the transcription of *hapA*, which encodes the secreted extracellular hemagglutinin (HA) protease. In addition, HapR represses the transcription of *aphA*, an upstream activator of *tcpP/H* transcription. TcpP/H and ToxR/S together activate *toxT* transcription, which in turn positively regulates virulence gene (e.g., CT and TCP) expression.

sequences were removed by standard sucrose counterselection, and sequencing of PCR products amplified from the chromosome was used to confirm that the single nucleotide change had been introduced.

The suicide plasmid encoding a transcriptional fusion of *lacZ* to the *hapR* promoter has been described (30) and was introduced into the chromosome of the indicated strains by homologous recombination (single crossover). The chromosomal *lacZ* gene was deleted from the strains by standard methods using pDLT (9), a derivative of pCVD442. The pACYC184-*toxS* plasmid has been described (22). A control plasmid (pACYC184) was obtained from New England Biolabs (Beverly, Mass.). The pBBR-*hapR* plasmid for expressing *hapR* has been described previously (30) and is a derivative of pBBR1-MCS4 (16). A control plasmid, identical to pBBR-*hapR* except that it expressed the nonfunctional *hapR* allele from *V. cholerae* N16961, was also generated. The *hapA* open reading frame (VCA0865, were encoding HA protease) and 302 bp of upstream sequence were amplified with the 5′ primer ATAGAATTCAAATAGTTGATAC ATCCTAGAAACTG and the 3′ primer ATACTCGAGGTACGTCAATCCC CTGTTGATACTG, digested with *Xho*I and *Eco*RI, and cloned into pBBR1-MCS4. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *hapR* expression plasmid pJZ146 has been described previously (30), and the corresponding control plasmid pMALc2x was obtained from New England Biolabs. All constructs were confirmed by sequencing.

Zymograms, milk plates, and azocasein protease assay. Zymograms were performed with cell-free culture supernatants as described previously (30). Briefly, 0.2% gelatin (Difco, Detroit, Mich.) was incorporated into a 7% polyacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was used to separate unboiled supernatant proteins. After the SDS was rinsed away, the in-gel protease activity, which resulted in clearing of the gelatin substrate, was detected by staining the gel with Coomassie blue. For milk plates, single colonies were patched onto LB agar plates containing 1% (wt/vol) nonfat powdered milk and allowed to grow overnight at 37°C. Protease activity was detected by clearing of the opaque milk proteins incorporated into the plate. The azocasein protease assay was performed as previously described (2). One azocasein unit is equal to the amount of enzyme releasing 0.01 optical density at 442 nm (OD_{442}) unit of colored substrate per h.

Luminescence. Overnight cultures (grown at 30°C in LB medium) of strains harboring the *V. harveyi luxCDABE* luciferase operon (carried on the pBB1 cosmid [21]) were diluted 1:1,000 and allowed to grow with aeration at 30°C. Luminescence was detected with a Berthold LB9507 luminometer.

Biofilm assays. Overnight cultures of *V. cholerae* were diluted into 1 ml of LB medium in autoclaved 12- by 75-mm borosilicate glass tubes and incubated at 22°C without agitation. After the indicated time, planktonic cells were washed away with distilled water and the remaining biofilm-associated cells were stained with 1% crystal violet. The tubes were rinsed three times and photographed. To quantify biofilm formation, stained biofilms were solubilized with 1 ml of dimethyl sulfoxide and OD at 570 nm determined.

RESULTS AND DISCUSSION

We sought to characterize a protease-deficient mutant of *V. cholerae* that had previously been isolated and designated E7946 *mpc.* Zymogram analysis demonstrates that E7946 *mpc* lacks expression of the secreted HA protease but still expresses several less active proteases (Fig. 2A). Southern blot analysis of E7946 *mpc* genomic DNA indicated that E7946 *mpc* harbors two Tn*5* insertions (data not shown). Arbitrary PCR suggested that one Tn*5* insertion is in the gene encoding ToxS, a well-characterized signaling protein essential for CT production by *V. cholerae* (22). Inverse PCR (13) was used to show that the second Tn*5* insertion is in VC2699 (*dcuA*), a putative transporter of C_4 dicarboxylic acids. Interestingly, however, extensive deletion and complementation analysis demonstrated that neither of the Tn*5* insertions (individually or together) were responsible for the protease defect of E7946 *mpc* (data not shown).

Previous work established that protease production in *V. cholerae* is under the control of a quorum-sensing signaling circuit (21, 30) (Fig. 1). We therefore hypothesized that E7946 *mpc* might harbor a novel mutation in a quorum-sensing gene. This hypothesis was supported by the observation that E7946 *mpc* exhibited a >1,000-fold defect in expression of the quorum-regulated luciferase operon (*luxCDABE*) transferred from *V. harveyi* (see below).

Since HapR is a critical activator of *hapA* (14) and controls other quorum-regulated genes of *V. cholerae* (30), we attempted to restore protease production in E7946 *mpc* with a *hapR* expression plasmid (pBBR-*hapR*). The pBBR-*hapR* plasmid only weakly restored protease production in E7946 *mpc* as assessed by zymogram analysis (Fig. 2A) and a quantitative azocasein protease assay (Fig. 2B). As a control, we found that pBBR-*hapR* significantly increased protease production by N16961, a *V. cholerae* strain that is a natural *hapR* mutant (30) (Fig. 2), and also fully restored protease production to *hapR* mutants of *V. cholerae* E7946 and C6706 (data not shown). These results, in combination with sequencing of the *hapR* gene from E7946 *mpc*, suggested that *mpc* is not a *hapR* mutant. A plasmid expressing *hapA* under the control of the *lac* promoter (pBBR-*hapA*) fully restored protease production by E7946 *mpc*, implying that E7946 *mpc* did not have a defect in protease translation or secretion (data not shown). The *hapA* gene of E7946 *mpc* was sequenced (including 170 bp of promoter sequence) and was found to be identical to that of wild-type E7946. Taken together, the above results suggested that the defect in E7946 *mpc* might be upstream of *hapR* and might result from a constitutively active allele of *luxO*, a known negative regulator of *hapR* (30).

We sequenced the *luxO* and *luxU* genes from E7946 and

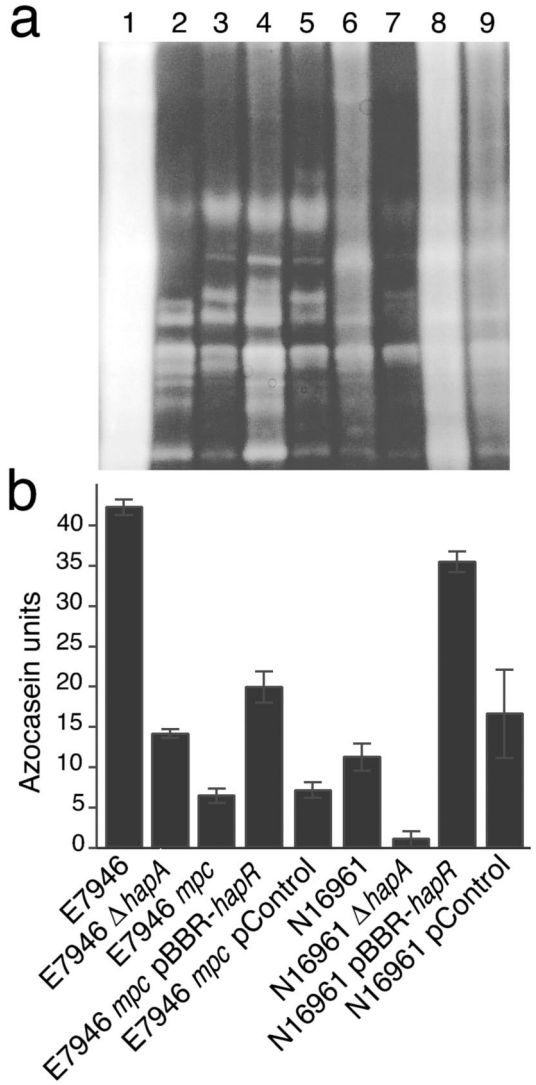

FIG. 2. Reduced extracellular protease activity in E7946 *mpc*. (A) Zymogram in-gel protease assay. Cell-free supernatants from overnight cultures of various strains were run on gelatin-impregnated SDS-PAGE gels. After separation of supernatant proteins, protease-mediated digestion of the gelatin was allowed to proceed and was detected by staining of the gel with Coomassie blue. Strains 1 to 9 are arranged in the same order as the strains in panel B: lane 1, E7946; lane 2, E7946 Δ*hapA*; lane 3, E7946 *mpc*; lane 4, E7946 *mpc* carrying pBBR-*hapR*; lane 5, E7946 *mpc* carrying pBBR-Control; lane 6, N16961; lane 7, N16961 Δ*hapA*; lane 8, N16961 carrying pBBR-*hapR*; lane 9, N16961 carrying pBBR-Control. In this assay, the HA protease (HapA) runs as a smear (compare E7946 and E7946 Δ*hapA*). pBBR-*hapR* is a plasmid expressing *hapR* from E7946. pBBR-Control is a similar plasmid but expressing the nonfunctional (frame-shifted) *hapR* from N16961. (B) Cell-free supernatants from overnight cultures of indicated strains were assayed for digestion of azocasein in triplicate. Error bars indicate standard deviation.

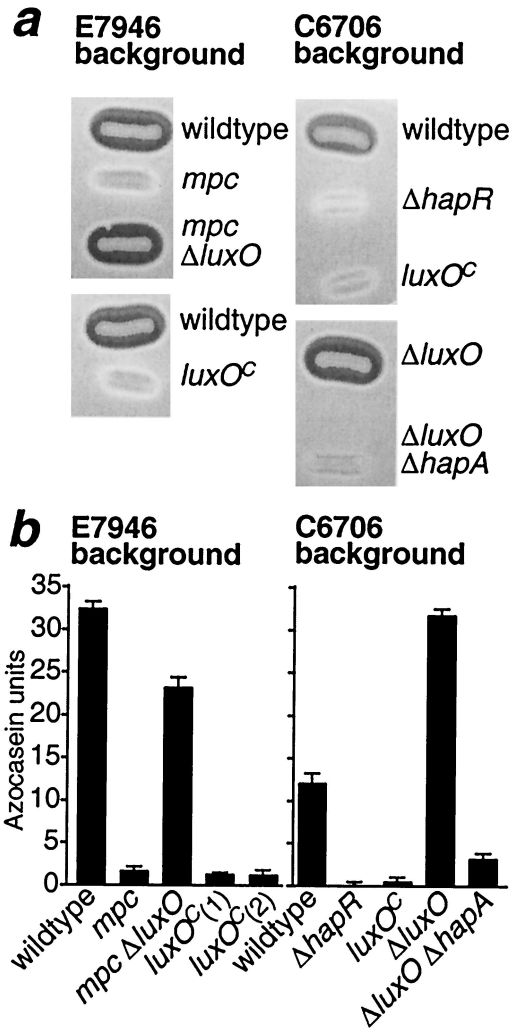

FIG. 3. (A) Milk plate assay for protease activity. Single colonies of the isolated strains were patched to agar plates containing 1% nonfat milk, incubated overnight at 37°C, and photographed against a black background. Protease activity is detected by clearing of the opaque milk proteins. *luxO*^{C} indicates the *luxO* allele from E7946 *mpc* (encoding the L104Q substitution). (B) Cell-free supernatants from cultures of indicated strains were assayed for digestion of azocasein in triplicate. Error bars indicate standard deviation. Two independent isolates of E7946 *luxO*(Con), designated *luxO*^{C}(1) and *luxO*^{C}(2), were tested.

E7946 *mpc* and discovered a single nucleotide change at nucleotide 311 of *luxO* (T to an A) that results in a leucine-to-glutamine change at amino acid 104 of the LuxO protein. We identified the LuxO protein from E7946 *mpc* as LuxO(Con). Work with *V. harveyi* has established that constitutively active alleles of *luxO* can be generated by mutation of the site at which LuxO is phosphorylated (e.g., D47E) or at a second site (e.g., F94W) which is thought to switch LuxO to an "open" and constitutively active conformation (7). The mutation observed in E7946 *mpc* maps near the second site and may therefore result in a constitutively active LuxO protein.

To prove that E7946 *mpc* expresses a constitutively active form of *luxO*, we conducted two experiments. First, we deleted *luxO*(Con) from the E7946 *mpc* background and found that the resultant E7946 *mpc* Δ*luxO* strain produces normal or even elevated levels of protease (Fig. 3). This finding confirms that the Tn*5* insertions in *toxS* and *dcuA* that are still harbored by E7946 *mpc* Δ*luxO* are probably not responsible for the protease-deficient phenotype. Second, we transferred the *luxO*(Con) allele into clean wild-type E7946 and C6706 backgrounds. The single point mutation in *luxO*(Con) was sufficient to eliminate protease production by E7946 and C6706 (Fig. 3).

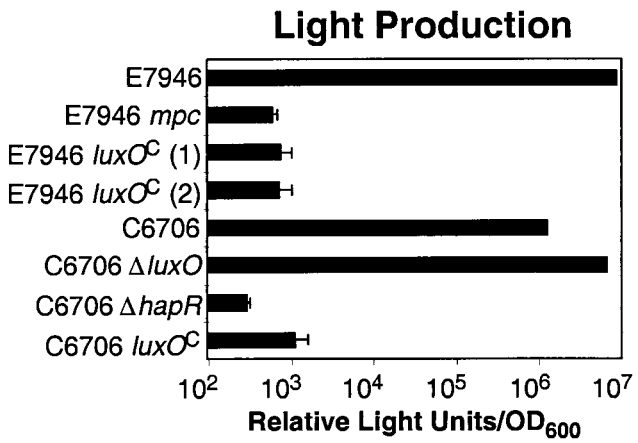

FIG. 4. Luminescence of *V. cholerae* strains harboring a cosmid containing the *luxCDABE* operon from *V. harveyi.* The indicated strains were diluted in triplicate and allowed to grow for 12 h at 30°C. The results are representative of three experiments. *luxO*^{C}, LuxO-(Con).

Taken together, our results demonstrate that the L104Q substitution in LuxO(Con) is a necessary and sufficient cause of the protease-deficient phenotype of E7946 *mpc.*

This conclusion was supported by an analysis of light production by various strains carrying the pBB1 cosmid (Fig. 4). pBB1 contains the luciferase (*luxCDABE*) operon from *V. harveyi*, and previous work has demonstrated that this operon is quorum regulated in *V. cholerae* (21). At high cell density (12 h of growth), wild-type E7946 and C6706 strains carrying pBB1 produce significant amounts of light whereas *luxO*(Con) mutants of E7946 or C6706 exhibit almost a 10,000-fold defect in light production (Fig. 4). Strains harboring *luxO*(Con) thus recapitulate the constitutively "dark" phenotype of *hapR* null mutants and exhibit a phenotype that is the converse of the constitutively "bright" *luxO* null mutants.

During the course of these analyses, we noted that overnight cultures of E7946 *mpc* often contained aggregated bacterial biomass, in contrast to E7946, which formed relatively uniform cell suspensions. We therefore suspected that the E7946 *mpc* strain might have an altered propensity for biofilm formation. We tested the ability of E7946 *mpc* to form a biofilm on the wall of a glass culture tube and found that E7946 *mpc* consistently formed thicker biofilms than did the parental E7946 strain (Fig. 5). To confirm that the biofilm phenotype was due to the mutation in *luxO* and not some other alteration in E7946 *mpc*, we tested derivatives of E7946 and C6706 harboring the constitutive *luxO*(Con) allele and found that these strains also formed thicker biofilms than did the wild-type strains (Fig. 5). *luxO*(Con) was necessary for the enhanced biofilm formation by E7946 *mpc*, since deletion of *luxO* from E7946 *mpc* eliminated biofilm formation. The phenotype of *luxO*(Con) strains resembled that of *hapR* mutant strains (Fig. 5) (30), as expected, since a constitutively active LuxO should always repress *hapR.*

The reason why *luxO* null mutants fail to produce significant biofilms remains under investigation. We tested the possibility that increased protease production in *luxO* mutants interfered with biofilm formation by examining biofilm formation in a

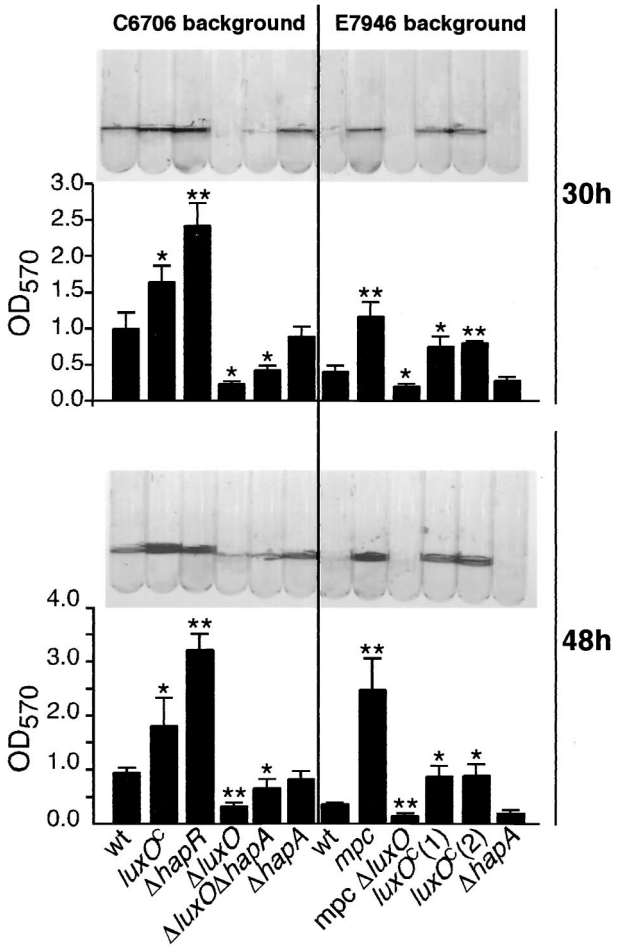

FIG. 5. Biofilm production on glass tubes in static cultures incubated at room temperature. After the indicated time, planktonic cells were rinsed away with distilled water, and the remaining adherent biofilm-associated cells were stained with crystal violet and photographed. The amount of biofilm formation for each strain was quantified by solubilizing the stained biofilm with DMSO and measuring the OD_{570}. Each strain was tested in quadruplicate at each time point. Error bars indicate standard deviation. A single asterisk (*) indicates that biofilm formation was significantly different ($P < 0.05$) from the corresponding parental wild-type (wt) strain, whereas a double asterisk (**) indicates a P of <0.005. *luxO*^{C} indicates the *luxO* allele from E7946 *mpc* (encoding the L104Q substitution). Two independent isolates of E7946 *luxO*(Con), designated *luxO*^{C}(1) and *luxO*^{C}(2), were tested. The results are representative of three experiments.

Δ*luxO* Δ*hapA* double mutant (Fig. 5). This strain resembled the single Δ*luxO* mutant and failed to produce normal biofilms, suggesting that overproduction of proteases is not solely responsible for the biofilm defect of *luxO* mutants; in addition, Δ*hapA* mutants of C6706 or E7946 did not produce significantly thicker biofilms than did their wild-type parental strains (Fig. 5). Therefore, it seems unlikely that enhanced biofilm formation by strains harboring the constitutive *luxO*(Con) allele is due solely to repression of protease production in these strains. Interestingly, Δ*luxO* Δ*hapA* mutants consistently produced slightly but significantly ($P < 0.05$) thicker biofilms than did Δ*luxO* mutants (Fig. 5). Thus, it remains possible that HA protease plays a minor role in biofilm formation, at least in the *luxO* mutant background. We are investigating whether exopo-

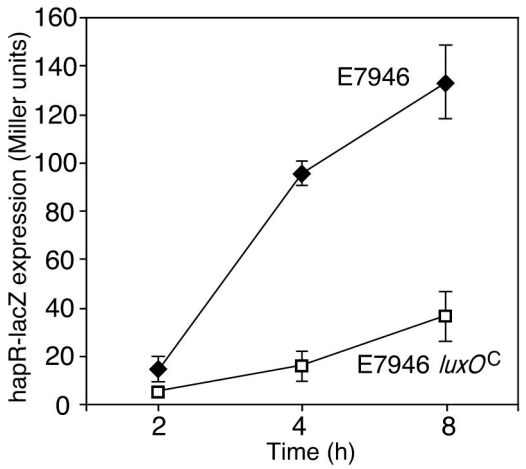

FIG. 6. *hapR* transcription is repressed by constitutive LuxO activity. The chromosomal *lacZ* gene was deleted from the indicated strains, and a transcriptional *hapR-lacZ* fusion was introduced at the chromosomal *hapR* locus. Overnight cultures were diluted into fresh LB medium and allowed to regrow, and samples were taken at the indicated time points for β-galactosidase assays (20). *luxO*^{C}, LuxO-(Con).

lysaccharide genes or other factors are the primary target of LuxO with respect to biofilm formation.

We wished to dissect further the mechanism by which LuxO interferes with the function of *hapR.* Results discussed above raised the possibility that LuxO might act posttranscriptionally on HapR, since expression of *hapR* from the pBBR-*hapR* plasmid failed to complement fully the E7946 *mpc* protease deficiency (although pBBR-*hapR* did correct a *hapR* deletion) (Fig. 2). In contrast, previous work (30) demonstrated that *luxO* deficiency resulted in increased *hapR* transcription at low cell densities. To test whether constitutive *luxO* acts transcriptionally on *hapR*, we transferred a *hapR-lacZ* transcriptional fusion into E7946 and E7946 *luxO*(Con) derivatives from which the chromosomal *lacZ* gene had been deleted. We found that the *hapR* gene was transcriptionally repressed in the strain harboring the LuxO(Con) protein (Fig. 6). The reason why the pBBR-*hapR* plasmid fails to restore protease production to *luxO*(Con) strains may be that this construct contains 200 bp of sequence upstream of the *hapR* start codon and that this 200 bp sequence may be the target of the transcriptional repression mediated by LuxO. This hypothesis is not yet conclusively demonstrated; however, it is worth noting that another *hapR* expression plasmid (pJZ146) (30), which lacks this 200-bp upstream sequence and instead drives *hapR* expression from a *tac* promoter, fully restored protease production to *luxO*(Con) strains (data not shown).

It should also be noted that although HA protease accumulates at high cell densities and is regulated by LuxO (30; also see above), we have not presented direct evidence demonstrating that HA protease expression is regulated by quorum sensing. In fact, a recent paper (2) demonstrated that supplementation of cultures with spent culture media (presumably containing autoinducers) had no effect on HA protease production by strain C7258. In contrast, *hapA* expression was induced by nutrient limitation and repressed by glucose (2), suggesting that expression of HA protease at high cell densities may be due to nutrient limitation rather than to the accumulation of quorum signals. Indeed, a thorough genetic analysis of the quorum-sensing systems in *V. cholerae* (21) suggests that nonquorum signals may feed into the LuxO/HapR signaling pathway.

Our results add to our understanding of the quorum-sensing signaling cascade in *V. cholerae.* This cascade appears to be central to a number of important virulence-related phenotypes, including CT production, protease secretion, and biofilm formation. Previous work (30) demonstrated that a *luxO* mutant formed poor biofilms, overproduced protease, and was avirulent in vivo. The present work complements and extends these findings by demonstrating that a constitutively active *luxO* allele enhances biofilm formation and inhibits protease production. We demonstrate that constitutively active *luxO* represses the transcription of *hapR.* The identification of a novel single amino acid substitution that leads to a constitutively active form of LuxO will be invaluable in understanding the structural basis of LuxO activation. Taken together, our results place LuxO as a central regulator that controls a switch between two distinct phenotypic phases of *V. cholerae*: in the "LuxO-ON" phase, *V. cholerae* is permissive for CT production, forms biofilms, and ceases protease production, whereas in the "LuxO-OFF" phase, biofilm formation is repressed, *hapR* is expressed and represses the ToxR regulon, and proteases are produced. It will be of interest to determine whether (and how) these phenotypic phases allow *V. cholerae* to survive in the environment and to colonize the human intestinal tract.

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