**Vibrio cholerae** ToxR Downregulates Virulence Factor Production in Response to Cyclo(Phe-Pro)

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**ABSTRACT** *Vibrio cholerae* is an aquatic organism that causes the severe acute diarrheal disease cholera. The ability of *V. cholerae* to cause disease is dependent upon the production of two critical virulence determinants, cholera toxin (CT) and the toxin-coregulated pilus (TCP). The expression of the genes that encode for CT and TCP production is under the control of a hierarchical regulatory system called the ToxR regulon, which functions to activate virulence gene expression in response to in vivo stimuli. Cyclic dipeptides have been found to be produced by numerous bacteria, yet their biological function remains unknown. *V. cholerae* has been shown to produce cyclo(Phe-Pro). Previous studies in our laboratory demonstrated that cyclo(Phe-Pro) inhibited *V. cholerae* virulence factor production. For this study, we report on the mechanism by which cyclo(Phe-Pro) inhibited virulence factor production. We have demonstrated that exogenous cyclo(Phe-Pro) activated the expression of *leuO*, a LysR-family regulator that had not been previously associated with *V. cholerae* virulence. Increased *leuO* expression repressed *aphA* transcription, which resulted in downregulation of the ToxR regulon and attenuated CT and TCP production. The cyclo(Phe-Pro)-dependent induction of *leuO* expression was found to be dependent upon the virulence regulator ToxR. Cyclo(Phe-Pro) did not affect toxR transcription or ToxR protein levels but appeared to enhance the ToxR-dependent transcription of *leuO*. These results have identified *leuO* as a new component of the ToxR regulon and demonstrate for the first time that ToxR is capable of downregulating virulence gene expression in response to an environmental cue.

**IMPORTANCE** The ToxR regulon has been a focus of cholera research for more than three decades. During this time, a model has emerged wherein ToxR functions to activate the expression of *Vibrio cholerae* virulence factors upon host entry. *V. cholerae* and other enteric bacteria produce cyclo(Phe-Pro), a cyclic dipeptide that we identified as an inhibitor of *V. cholerae* virulence factor production. This finding suggested that cyclo(Phe-Pro) was a negative effector of virulence factor production and represented a molecule that could potentially be exploited for therapeutic development. In this work, we investigated the mechanism by which cyclo(Phe-Pro) inhibited virulence factor production. We found that cyclo(Phe-Pro) signaling through ToxR to activate the expression of *leuO*, a new virulence regulator that functioned to repress virulence factor production. Our results have identified a new arm of the ToxR regulon and suggest that ToxR may play a broader role in pathogenesis than previously known.
teria neither the biological function nor the mechanism of CDP biosynthesis is known. CDPs have been reported to modulate the expression of genes controlled by LuxR-type quorum sensing (QS) systems (14–16), a finding that suggested that CDPs may function as bacterial signaling molecules (16). Consistent with this idea, Vibrio spp. produce cyclo(Phe-Pro) (cFP), a CDP that functions as an effector of V. cholerae virulence gene expression (17, 18). cFP accumulates in V. cholerae culture supernatants in a growth-dependent manner and reaches a maximum concentration of ~0.8 mM as the cultures transition to stationary-phase growth (17). Work in our laboratory previously showed that the addition of cFP to cultures grown under virulence gene-inducing conditions inhibited tcpP expression and attenuated CT and TCP production (18). These results, coupled with the observation that cFP activated the expression of the ToxR-regulated omptU porin (17, 18), suggested that cFP may be affecting virulence gene expression via the ToxR regulon.

In this study, we sought to elucidate the cFP-dependent signal transduction cascade that led to attenuated virulence gene expression. The results of this work revealed that cFP activated a novel ToxR-dependent signaling cascade. Our results showed that cFP signaled through ToxR to activate leuO expression. LeuO is a LysR-family DNA binding protein that functions as a global regulator in other enteric pathogens (19, 20) but had not been previously associated with virulence in V. cholerae. Upregulation of leuO resulted in repressed aphA transcription which led to down-regulation of the ToxR regulon and attenuated CT and TCP production. Our results have identified a new branch of the ToxR regulon and show that ToxR is capable of downregulating virulence factor production in response to an environmental cue.

RESULTS

cFP inhibits CT and TCP production in multiple epidemic V. cholerae strains. Previous analysis showed that cFP inhibited CT and TCP production in O1 El Tor strain N16961. We therefore tested cFP for inhibitory activity against two additional epidemic El Tor strains (21, 22). The respective strains were grown overnight under AKI conditions in the presence or absence of 1 mM cFP before being processed for CT and TcpA quantification. The results showed that cFP inhibited CT and TCP production in strains HK1 and MO10 to a similar (HK1) or greater (MO10) extent than was observed in N16961 (see Fig. S1 in the supplemental material). These data suggest that cFP inhibition of virulence factor production is not a strain-specific phenomenon and is conserved among a broad range of V. cholerae strains.

cFP induces leuO transcription. Previous work in our laboratory showed that cFP inhibited tcpP expression (18). This suggested that cFP might be affecting genes that are upstream of tcpP in the ToxR regulon. To identify upstream genes, we performed microarray analysis of V. cholerae grown under AKI conditions in the presence and absence of cFP. Gene expression was assayed at 3 h in order to determine the effect of cFP on genes that were induced prior to tcpP. The microarray analysis resulted in the identification of 18 genes that were differentially expressed in the presence of cFP (see Table S1 in the supplemental material). This is a conservative estimate of cFP-responsive genes, since known cFP-responsive genes (e.g., tcpPH and the genes involved in CT and TCP biosynthesis) were not identified as differentially expressed. The omission of these genes was likely due to a combination of experimental variability and the early time point used for analysis. Two of the differentially expressed genes, vexR and leuO, encoded regulatory proteins that could be involved in cFP signaling. VexR appears to be a regulator of the VexAB RND efflux system and was not considered for further study here (23, 24). In contrast, leuO was deemed a high-priority gene for follow-up due to its role as a global regulator in other bacteria (19, 20).

The effect of cFP on leuO was confirmed by quantitative reverse transcription-PCR (qRT-PCR). Wild-type (WT) V. cholerae was grown under AKI conditions in the presence and absence of cFP, and RNA was isolated at 3 h and 6 h. These two time points were selected to confirm that changes in gene expression were not growth phase dependent. The results confirmed the microarray data and showed that cFP induced leuO expression by 2.0-fold at 3 h and 4.1-fold at 6 h (Fig. 1). cFP also repressed tcpP expression at 3 h and 6 h by 3.4- and 4.6-fold, respectively, confirming our published results (18). Since cFP inhibited tcpP expression, we tested if cFP affected aphA and aphB expression. ToxR was included as a control since it appears to be constitutively expressed under most growth conditions. The results showed that cFP did not appreciably affect aphB or toxR expression (Fig. 1). In contrast, cFP inhibited aphA transcription by 2.1-fold at 3 h and 2.8-fold at 6 h. This suggested that cFP likely effected tcpP expression by downregulating aphA. Previous analysis using a plasmid-based reporter did not reveal cFP repression of aphA (18). This discrepancy may have resulted from plasmid copy number effects or other regulatory mechanisms that are known to affect aphA (25).

LeuO represses CT and TCP production. Since leuO was induced by cFP, we sought to determine if leuO functioned in virulence regulation. We therefore tested the effect of leuO expression on CT and TCP production. The WT containing pBAD18-leuO or the pBAD18 control was grown under AKI conditions in the presence of 0.02% or 0.08% arabinose before being assayed for CT and TcpA production. The results showed that leuO overexpression attenuated CT and TcpA production (Fig. 2A). These findings suggested that LeuO was a CT and TCP repressor.

LeuO is encoded in an apparent operon consisting of VC2486 and VC2485 (leuO) (Fig. 2C). VC2486 is a 102-bp open reading frame among a broad range of V. cholerae strains. Previous analysis showed that cFP inhibited CT and TCP production in O1 El Tor strain N16961. We therefore tested cFP for inhibitory activity against two additional epidemic El Tor strains (21, 22). The respective strains were grown overnight under AKI conditions in the presence or absence of 1 mM cFP before being processed for CT and TcpA quantification. The results showed that cFP inhibited CT and TCP production in strains HK1 and MO10 to a similar (HK1) or greater (MO10) extent than was observed in N16961 (see Fig. S1 in the supplemental material). These data suggest that cFP inhibition of virulence factor production is not a strain-specific phenomenon and is conserved among a broad range of V. cholerae strains.

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from VC2486 in an apparent two-gene operon. VC2486 and leuO consensus sequences in the V. cholerae leuO (pBAD18) or WT (pBAD18-ELISA and TcpA Western blotting. (A) CT and TcpA production in the WT representative of three experiments /H11006 (B) CT and TcpA production in the WT and the frame (ORF) located upstream of FIG 2

that of the control during growth under AKI conditions (Fig. 3C). Expression ratio

Based on these results, we concluded that LeuO was an aphA repressor.

leuO deletion did not affect CT or TcpA production (Fig. 2B).

LeuO represses aphA expression. We tested if LeuO affected aphA expression by using qRT-PCR to compare aphA expression in the WT and a ΔleuO mutant. The results showed a 2.4-fold increase in aphA expression and a 2.7-fold increase in tcpP expression in the ΔleuO strain relative to that in the WT (Fig. 3A). The fact that leuO deletion resulted in increased tcpP expression indicated that LeuO was an aphA repressor. We then overexpressed leuO in the WT under AKI conditions and quantified aphA, aphB, tcpP, and toxR expression by qRT-PCR. This showed that leuO overexpression resulted in a 4.4-fold decrease in aphA expression and a 6.6-fold decrease in tcpP transcription (Fig. 3B), further confirming that LeuO was an aphA repressor. In contrast, leuO overexpression did not have a significant effect on aphB or toxR. Overexpression of leuO also inhibited TcpP production relative to that of the control during growth under AKI conditions (Fig. 3C).

FIG 2 Effect of leuO on CT and TcpA production. The indicated strains were grown overnight under AKI conditions, normalized by OD, and used for CT ELISA and TcpA Western blotting. (A) CT and TcpA production in the WT (pBAD18) or WT (pBAD-leuO) strain grown with 0.02% or 0.08% arabinose. (B) CT and TcpA production in the WT and the ΔleuO mutant. The results are representative of three experiments ± SD. (C) Schematic diagram of the V. cholerae leuO locus. The gene encoding LeuO (VC2485) is downstream from VC2486 in an apparent two-gene operon. VC2486 and leuO are separated by 15 nucleotides (nt) and appear to be expressed from a common promoter that is located upstream of VC2486. The two ToxR-binding site consensus sequences in the leuO promoter are indicated by the yellow boxes.

FIG 3 Effect of leuO on aphA and tcpP expression. (A) Quantification of gene expression by qRT-PCR. The WT or ΔleuO strain grown under AKI conditions. (B) Effect of leuO on the expression of the indicated genes. The WT (pBAD18) and WT (pBAD18-leuO) strains were grown under AKI conditions for 3 h, when RNA was isolated and used for qRT-PCR. The means and SEM for three biological replicates are presented. *, P < 0.01 from a hypothetical value of 1.0. (C) TcpP Western blot of WT(pBAD18) and WT(pBAD18-leuO) grown under AKI conditions with 0.02% arabinose. Aliquots were collected at the indicated times and normalized by OD before analysis. (D) TcpP Western blot of the WT and ΔleuO strains grown under AKI conditions for the indicated times. The samples were processed as described above. Samples presented within panels C and D were run on the same gel and exposed to X-ray film for identical times. The resulting X-ray film images were then converted to grayscale TIFF files, and the contrast and brightness were adjusted before being cropped to generate panels C and D.

Cyclo(Phe-Pro) Signals through ToxR

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results show that *leuO* is a major factor in the cFP-dependent inhibition of virulence factor expression. However, the fact that *leuO* deletion did not completely abrogate cFP activity suggests that cFP also affects virulence factor production by a *leuO*-independent mechanism.

**ToxR is required for *leuO* expression.** cFP was previously shown to induce expression of the ToxR-regulated *ompU* porin (17, 18). This suggested that ToxR may function in cFP signaling and possibly *leuO* regulation. We tested this hypothesis by quantifying *leuO-lacZ* expression in WT, Δ*toxRS*, Δ*toxT*, and ΔtcpPH strains during growth under AKI conditions. The results showed that *leuO* was expressed at a low level in the WT at 3 h and that its expression increased with cell growth and reached a maximum expression level at stationary phase (Fig. 5A). Expression of *leuO* in the Δ*toxT* and ΔtcpPH mutants was similar to that in the WT, suggesting that neither of these genes affects *leuO*. In contrast, *leuO* expression in the Δ*toxRS* mutant was dramatically reduced relative to that in the WT, indicating that ToxR was a positive regulator of *leuO* and that ToxR was responsible for the growth-dependent upregulation of *leuO*. Although *leuO* expression was dramatically reduced in the *toxR* mutant, *leuO* was still expressed at a low basal level and was induced at stationary phase. This suggests that other factors in addition to ToxR likely contribute to *leuO* expression.

**ToxR is required for cFP induction of *leuO* expression.** Our data indicated both cFP and ToxR activated *leuO* expression. We therefore tested if cFP activity was dependent on ToxR by quantifying *leuO* and *aphA* expression in the WT and the Δ*toxRS* mutant during growth in the presence and absence of cFP. The results showed that the addition of cFP caused a 4.2-fold increase in *leuO* expression in the WT (Fig. 5B). In contrast, cFP did not have a significant effect on *leuO* expression in the *toxRS* mutant. Likewise, the addition of cFP caused a 2.8-fold decrease in *aphA* expression in the WT but did not significantly affect *aphA* expression in the *toxRS* mutant. Taken together, these results strongly suggest that cFP signals through ToxR to activate *leuO* expression and to repress *aphA* expression.

Since our data suggested that cFP did not affect toxR expression (Fig. 1), we hypothesized that cFP may affect the ability of ToxR to activate *leuO* transcription. If this was true, we posited that cFP would enhance *leuO* expression in a complemented Δ*toxRS* strain. We therefore tested the effect of cFP on *leuO* expression in the Δ*toxRS* strain DT730 containing pBAD18::toxRS or the control vector (pBAD18) during growth under AKI conditions in the presence of arabinose. The results showed that toxRS expression complemented DT730 for *leuO* expression in the absence of cFP as exhibited by the 25.6-fold increase in *leuO* expression relative to that for the vector control (Fig. 5C); this further confirmed that ToxR was a positive regulator of *leuO*. The addition of cFP to the AKI broth caused an ~2-fold increase in *leuO* expression relative to that of the same strains grown without cFP (Fig. 5C); this further confirmed that cFP enhanced the ability of ectopically expressed ToxR to activate *leuO* expression. Collectively these data suggest that the cFP-dependent induction of *leuO* expression was likely a result of cFP stimulation of ToxR transcriptional activity.

The above data did not exclude the possibility that cFP also increased the cellular ToxR pool. We therefore determined the effect of cFP on ToxR production during growth under AKI conditions. The results showed that cFP did not affect ToxR production relative to that of the WT or the dimethyl sulfoxide (DMSO) control at any time point (Fig. 5D). The amount of ToxR in the cells did appear to increase during growth. An increase in ToxR was apparent between 4 and 6 h, which correlated with *leuO* in
duction (Fig. 5A). This suggests that the growth-dependent increase in ToxR could contribute to the growth-dependent increase in leuO expression.

The periplasmic domain of ToxR is required for cFP induction of leuO expression. We tested the hypothesis that the ToxR periplasmic domain (PPD) was involved in cFP signaling. This was accomplished by determining whether loss of the ToxR PPD affected cFP activity. These experiments used a mutant version of ToxR, called ToxR\textsuperscript{mem}, that lacked the PPD. Previous studies have shown that the PPD deletion in ToxR\textsuperscript{mem} did not affect ToxR regulon expression during growth under AKI conditions (30).

We first confirmed that ToxR\textsuperscript{mem} was functional in *V. cholerae* by testing whether ectopic expression of toxR\textsuperscript{mem} could complement a ΔtoxRS mutant for OmpU production. We cultured WT and ΔtoxRS strains containing pBAD18, pBAD18-toxRS, or pBAD18-toxR\textsuperscript{mem} under AKI conditions in the presence and absence of arabinose. Following overnight growth, normalized aliquots from each culture were resolved by SDS-PAGE. Porin production was then visualized by Coomassie brilliant blue dye staining and by OmpU Western blotting. The results showed that WT *V. cholerae* produced only OmpU, whereas the complemented ΔtoxRS strains grown in the absence of arabinose produced only OmpT (see Fig. S2 in the supplemental material). This was expected, since ToxR is an ompU activator and ompT repressor (31, 32). The addition of arabinose to the AKI broth resulted in a shift from OmpT to OmpU production in the ΔtoxRS strains containing pBAD18-toxRS and pBAD18-toxR\textsuperscript{mem}, while the empty vector control (i.e., pBAD18) produced only OmpT (see Fig. S2). These data confirmed the previously published data (30) and showed that ToxR\textsuperscript{mem} is functional and can complement for loss of ΔtoxRS during growth under AKI conditions.

We next tested whether ToxR\textsuperscript{mem} affected cFP signaling. We cultured the ΔtoxRS strain containing pBAD18, pBAD18-toxRS, or pBAD18-toxR\textsuperscript{mem} under AKI conditions (plus arabinose) in the presence and absence of cFP for 4 h and assayed for leuO and tcpP expression by qRT-PCR. The results showed that cFP induced leuO expression and repressed tcpP expression in the WT (Fig. 6) and that cFP activity was lost in the ΔtoxRS (pBAD18) control strain. The presence of pBAD18-toxRS restored cFP-dependent effects on leuO and tcpP expression in the ΔtoxRS mutant, confirming that toxRS was required for cFP signaling. In contrast, the presence of pBAD18-toxR\textsuperscript{mem} in the ΔtoxRS strain failed to restore cFP activity. This finding suggests that the periplasmic domain of ToxR is required for cFP activity.

ToxR consensus binding sites are present in the leuO promoter. ToxR regulates the expression of many genes by binding to a consensus sequence located in the promoter of the target genes (33). Analysis of the leuO promoter showed the presence of two ToxR consensus sequence sites, located at −126 to −112 and −104 to −90, that were 100% conserved with the published consensus sequence (Fig. 7A). The presence of the ToxR consensus sequences in the leuO promoter was consistent with ToxR being a leuO activator and suggested that ToxR may function directly at the leuO promoter. To test this possibility, we expressed toxRS in the presence of a leuO-lux reporter (pJB906) in *Escherichia coli*. The results showed that in the absence of arabinose, leuO-lux was expressed at a low basal level (Fig. 7B). However, when toxRS expression was induced, we saw a dramatic arabinose dose-dependent increase in leuO-lux expression. Taken together, these results are consistent with ToxR acting directly on the leuO promoter. We were unable to test if cFP affected toxRS activation of leuO-lux expression since cFP inhibits luminescence production in vitro.

Expression of leuO in vivo. We tested the ΔleuO mutant in the infant mouse competition model to determine if leuO affected colonization. The results showed that the ΔleuO mutant competed equally with the WT for colonization, which indicated that leuO was dispensable for colonization (see Fig. S3A in the supplemental material). This finding is consistent with the apparent function of leuO as a virulence repressor that functions late in infection. To confirm that leuO was expressed in vivo, we challenged infant mice with *V. cholerae* containing pJB906 (leuO-lux) and imaged bioluminescence production in the mice following overnight incubation. The results showed luminescence production in the gut of mice challenged with *V. cholerae* bearing the leuO-lux reporter, whereas there was no luminescence production in mice challenged with the empty vector control (pCM10) (see Fig. S3B). From this, we concluded that leuO was expressed in the infant mouse small intestine during colonization.

**DISCUSSION**

The ToxR regulon has been a focus of cholera research for more than three decades. From this work, a model has emerged whereby the ToxR regulon functions to activate virulence gene expression in response to *in vivo* stimuli. In this work, we showed for the first time that the ToxR regulon can also repress virulence factor production. Our data, in conjunction with published results (17, 18), support a model where ToxR functions in a novel regulatory circuit that links cFP to virulence gene expression. We showed that in response to extracellular cFP, ToxR significantly upregulated leuO...
expression. LeuO then downregulated the ToxR regulon by repression of *aphA* expression.

The role of ToxR in cFP signaling was evidenced by the findings that *toxR* deletion decreased *leuO* expression (Fig. 5A) and abolished cFP-dependent induction of *leuO* expression (Fig. 5B) and that cFP activated *leuO* transcription by ectopically expressed toxR in a complemented *toxR* mutant (Fig. 5C and 6). Taken together, these results strongly suggested that cFP signaled through ToxR to upregulate *leuO* expression. The linkage of ToxR to cFP signaling is significant, since cFP is unlikely to enter *E. coli* by hydrophobic uptake. Therefore, *V. cholerae* requires a means to sense cFP in the external environment. ToxR is a protein that could fulfill this role. ToxR is a membrane protein that contains a periplasmic sensing domain that is linked to a cytoplasmic DNA binding domain by a transmembrane spanning domain (7). The orientation of ToxR in the membrane is thought to facilitate the transduction of environmental stimuli across the cytoplasmic membrane to modulate the expression of ToxR-regulated genes (34). Consistent with this, a number of extracellular stimuli have been shown to modulate ToxR-regulated genes (35). This fact adds significance to our finding that cFP activity was dependent upon the ToxR PPD.

The observation that cFP activity was dependent upon the ToxR PPD was consistent with the presumed role of the PPD in signal transduction. However, the mechanism by which cFP affects ToxR is unclear. The function of the ToxR PPD in gene regulation is not entirely clear. The PPD was found to be dispensable for *ompT* and *ompU* regulation and virulence gene expression during growth under virulence gene-inducing conditions (30). In contrast, the PPD was required for proper porin expression during growth in minimal medium via a process that was linked to disulfide bond formation (36). The latter finding is consistent with the hypothesis that the PPD functions in environmental sensing. It is tempting to speculate that cFP may interact directly with ToxR via the PPD; however, we cannot rule out the possibility that cFP acts indirectly on ToxR through other proteins or by effects on the cytoplasmic membrane. Work is ongoing to discriminate between these possibilities.

The requirement for ToxR in cFP signaling suggests that ToxR may have broader functions in pathogenesis than was previously known. The current model for the ToxR regulon shows that ToxR plays an essential role in activating virulence factor production early in infection (2). The work presented here shows that ToxR could also play a role in downregulating virulence factor production. Studies in humans and animals have shown that the genes responsible for CT and TCP production were poorly expressed late in infection (9–11, 37), which indicated the existence of a mechanism to downregulate CT and TCP production late in infection. While the quorum sensing systems and ToxT and TcpP proteolysis have been implicated in this process (27, 29, 38), we hypothesize that the ToxR-dependent mechanism described in this work could also contribute to this phenotype. This hypothesis is supported by the observation that *leuO* was expressed in the infant mouse intestine (see Fig. S3 in the supplemental material), a finding that was consistent with published data indicating that the *leuO* operon was also expressed in human-shed *V. cholerae* (9) and in rabbit ileal loops (11, 39). Late in infection, *V. cholerae* also appears to be exposed to conditions under which cFP could be relevant (17, 18). For example, *V. cholerae* titer in the intestinal lumen can exceed 10⁸ CFU/ml late in infection, and human- and animal-derived *V. cholerae* exhibits genetic signatures that are consistent with stationary-phase cells (9–11, 37). While these data are suggestive, additional work will be required to determine if ToxR is affecting the expression of genes late in infection.

LeuO had not been previously implicated in *V. cholerae* virulence. LeuO was first identified as a leucine operon activator in *Salmonella* (40). Subsequently, *leuO* was shown to function as a stationary-phase global regulator that affected the expression of multiple phenotypes, including virulence (19). We speculate that LeuO functions similarly in *V. cholerae*. This conclusion is supported for the following observations: *leuO* was induced at stationary phase (Fig. 5A), *leuO* overexpression inhibited virulence factor production (Fig. 2A), and *leuO* was associated with *V. cholerae* biofilm production (41). It is interesting to note that in *E. coli* LeuO functioned to regulate the expression of many genes by H-NS antagonism (20). This does not appear to be the case with *V. cholerae* virulence, since H-NS is also a virulence repressor (42–44). It appears that the role of LeuO in virulence may extend to other *Vibrio* spp., since recent reports have linked toxR and *leuO* to production of the type III secretion system in *Vibrio parahaemolyticus* (45, 46). Given that *V. parahaemolyticus* also produces cFP (17), it is possible that the ToxR-dependent cFP signaling pathway described in this work also functions in *V. parahaemolyticus*.
The cFP-dependent system appears to be redundant with the V. cholerae QS systems for virulence regulation, since both systems function to repress apnA. It is interesting to speculate that the cFP-dependent system could function late in infection as an alternative method to downregulate the ToxR regulon in QS-negative strains that are widespread among toxigenic V. cholerae isolates (22, 47). The similarity between the cFP-dependent system and the QS systems leads to the question of whether the cFP-dependent system is a QS system. This is unclear at present because we have not yet been able to identify the gene(s) responsible for cFP biosynthesis. In the absence of a cFP-negative mutant, we cannot determine whether cFP functions as a diffusible signal. We also cannot rule out the alternative hypothesis that cFP functions as an intracellular cue. For example, it is possible that cFP is produced in response to the metabolic status of the cell (e.g., amino acid limitation). This would be consistent with the induction of leuO at stationary phase and the reported roles of ToxR and LeuO in responding to environmental stimuli, including specific amino acids (19, 48, 49). This scenario could also explain leuO expression in vivo, since both human- and animal-derived V. cholerae bacteria exhibit genetic signatures indicative of growth in a nutrient-limiting environment (9, 11, 37).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table S2 in the supplemental material. Strain JB58 (V. cholerae O1 El Tor strain N16961 ΔlacZ Smr) was used as the wild type (WT) for all experiments unless noted otherwise. E. coli EC1004pir and SM10apor were the hosts for plasmid construction and conjugation, respectively. All bacterial strains were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. V. cholerae was grown under AKI conditions for induction of the ToxR regulon as previously described (50). Antibiotics were used at the following concentrations: carbenicillin (Cb), 100 μg/ml; kanamycin (Km), 50 μg/ml; streptomycin (Sm), 100 μg/ml. Synthetic CFP (Bachem, Bubendorf, Switzerland) was used for all experiments and was dissolved in DMSO. Arabinose was added to media at the indicated concentrations to induce expression from the arabinose-regulated promoter in pBAD18 and pBAD18Km.

Plasmid and mutant construction. V. cholerae O1 El Tor strain N16961 genomic DNA was used as a template for PCR amplification and cloning of all V. cholerae genes and gene promoters. Expression plasmid pXB269 was constructed by cloning the V. cholerae leuO locus into the multiple cloning site of pBAD18Km by PCR using the pBAD-leuO-F and pBAD-leuO-R primers (see Table S2 in the supplemental material). pXB297 and pXB298 were generated, respectively, by self religation of pXB269 that had been digested with SphI/EcoRI or XcmI/EcoRI. The toxRS expression plasmid pXB289 was created by cloning the toxRS gene into pBAD18 using the pBAD-toxRS-F and pBAD-toxRS-R primers. The lacZ fusion plasmid pXB266 was created by cloning the leuO promoter into pTL161T using the P_{lacA'}::F/P_{lacO} R primers (see Table S2). The lux reporter plasmid pB906 was also created using the P_{lacO}::F/P_{lacO} R PCR amplicon, which was digested with EcoRI/BamHI and cloned into the same restriction site in pCM10 to generate pJB906. The leuO deletion plasmid pWM91ΔleuO was used as previously described (41) to create strain XBV222. Plasmid pXR286 was constructed by crossover PCR as previously described using the respective pBAD-toxR_{emo} PCR primers listed in Table S2 (51, 52).

Method for microarray analysis. V. cholerae strain JB58 was grown under AKI conditions in the presence and absence of 1 mM cFP for 3 h when total RNA was purified from the cultures using Trizol (Invitrogen) according to the manufacturer’s instructions. The resulting RNA was treated with DNase and further purified on an RNeasy column according to the manufacturer’s directions (Qiagen) before being used for the microarray profiling experiments. The microarrays were provided by the Pathogen Functional Genomics Resource Center (Rockville, MD) and consisted of glass slides with 3,754 V. cholerae genes represented by 70-bp oligonucleotides that were spotted in triplicate. Processing of the mRNA and hybridization to the microarrays were performed as described at http://pfgrc.jcvi.org/index.php/microarray/protocols.html. A total of four microarrays were performed, including two dye swap experiments. The resulting microarrays were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA), and the data were analyzed using the TM4 microarray suite (J. Craig Venter Institute). The scanned images were first processed using TM4 Spotfinder software using local background subtraction to generate the intensity data for each probe. The resulting data were normalized in the TM4 MIDAS program using the default normalization settings with a low-intensity filter of 75. Significant genes were then identified from the MIDAS output by applying a one-class t test analysis using the TM4 MeV software tool, and significant genes exhibiting a change in expression of ≥1.5-fold were selected for further analysis.

Quantitative real-time PCR, gene reporter assays. V. cholerae cultures were grown under AKI conditions for virulence regulation, since both systems for virulence regulation, since both systems lead to the question of whether the cFP-dependent system is a QS system. This is unclear at present because we have not yet been able to identify the gene(s) responsible for cFP biosynthesis. In the absence of a cFP-negative mutant, we cannot determine whether cFP functions as a diffusible signal. We also cannot rule out the alternative hypothesis that cFP functions as an intracellular cue. For example, it is possible that cFP is produced in response to the metabolic status of the cell (e.g., amino acid limitation). This would be consistent with the induction of leuO at stationary phase and the reported roles of ToxR and LeuO in responding to environmental stimuli, including specific amino acids (19, 48, 49). This scenario could also explain leuO expression in vivo, since both human- and animal-derived V. cholerae bacteria exhibit genetic signatures indicative of growth in a nutrient-limiting environment (9, 11, 37).

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plated on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to verify the input ratio of the two strains. The microarray data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE38272.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org.

Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Figure S1, EPS file, 1.2 MB.
Figure S2, EPS file, 2.2 MB.
Figure S3, EPS file, 1.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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