The VarS/VarA two-component system modulates the activity of the *Vibrio cholerae* quorum-sensing transcriptional regulator HapR

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The human pathogen *Vibrio cholerae* uses quorum sensing to regulate the expression of a number of phenotypes, including virulence factor production, in response to changes in cell density. It produces small molecules called autoinducers that increase in concentration as cell density increases, and these autoinducers bind to membrane sensors once they reach a certain threshold. This binding leads to signalling through a downstream phosphorelay pathway to alter the expression of the transcriptional regulator HapR. Previously, it was shown that the VarS/VarA two-component system acts on a component of the phosphorelay pathway upstream of HapR to regulate HapR expression levels. Here, we show that in addition to this mechanism of regulation, VarS and VarA also indirectly modulate HapR protein activity. This modulation is mediated by the small RNA CsrB but is independent of the known quorum-sensing system that links the autoinducers to HapR. Thus, the VarS/VarA two-component system intersects with the quorum-sensing network at two levels. In both cases, the effect of VarS and VarA on quorum sensing is dependent on the Csr small RNAs, which regulate carbon metabolism, suggesting that *V. cholerae* may integrate nutrient status and cell density sensory inputs to tailor its gene expression profile more precisely to surrounding conditions.

**INTRODUCTION**

Pathogenic bacteria must possess intricate sensory and regulatory pathways to effect global changes in gene expression during transitions between starkly contrasting living conditions, such as those between environmental reservoirs and host organisms. Even within their environmental reservoir or within their host, living conditions can vary drastically. Two-component signalling systems are a common method used by bacteria to sense external signals, transmit information intracellularly, and convert this information into an appropriate change in gene expression. These systems typically involve a sensor kinase, located in the cell membrane, which detects environmental cues and phosphorlates a response regulator. Phosphorylation of the response regulator then alters its activity, which can include DNA binding and transcriptional control, among other functions (Gao *et al.*, 2007; Mitrophanov & Groisman, 2008).

*Vibrio cholerae* is commonly found in estuarine environments, and it is responsible for the severely dehydrating diarrhoeal disease cholera. Quorum sensing is one mechanism used by *V. cholerae* to translate changes in environmental conditions, specifically changes in cell density, into changes in gene expression. *V. cholerae* senses cell density by producing and detecting small molecules known as autoinducers that accumulate with increasing cell density. The autoinducers are sensed by two-component signalling systems that transmit information downstream by using a phosphorylation cascade. At low cell density, the cognate sensor kinases for the autoinducers, CqsS and LuxPQ, are unbound, and they phosphorylate LuxU, which in turn passes the phosphate on to LuxO (Miller *et al.*, 2002) (Fig. 1). Phosphorylated LuxO then activates the transcription of four small regulatory RNAs that destabilize the *hapR* mRNA transcript (Lenz *et al.*, 2004). Conversely, when autoinducers accumulate at high cell density, they bind their cognate sensors and cause conformational changes that lead to dephosphorylation of LuxO (Neiditch *et al.*, 2006). Dephosphorylated LuxO no longer activates transcription of the regulatory small RNAs, so the *hapR* transcript is stabilized. Due to this regulatory pathway, HapR is only expressed at high enough levels to mediate its downstream effects at high cell density. HapR is a TetR-family transcriptional regulator that has been shown to regulate a variety of phenotypes important for both virulence and environmental survival. It is capable of activating the transcription of the *luxCDABE* operon from *Vibrio harveyi*, which is useful as a reporter for HapR activity, and it is also able to activate transcription of *hapA*, which is important for virulence factor production.
which encodes the endogenous HapA protease (Jobling & Holmes, 1997; Miller et al., 2002). HapR has also been shown to repress biofilm formation and virulence factor production (Hammer & Bassler, 2003; Miller et al., 2002; Zhu et al., 2002; Zhu & Mekalanos, 2003). We have used a bioinformatics-based approach to identify novel targets of HapR (Tsou et al., 2009), and one of the newly identified targets, vca0880, is used as a reporter for HapR activity in the present study.

Another two-component system known to regulate virulence in V. cholerae is the VarS/VarA signalling system, and varA mutants have been shown to be attenuated in mice (Wong et al., 1998). VarS/VarA homologues are found in other bacteria, including BarA/UvrY in Escherichia coli and GacS/GacA in Pseudomonas species, and these signalling systems are often involved in virulence in these other bacteria as well (Heeb & Haas, 2001; Herren et al., 2006; Pernestig et al., 2001; Tomenius et al., 2006). The signals that activate VarS/VarA-type systems have not yet been definitively established. VarS and VarA activate the transcription of csrB, csrC and csrD, which encode untranslated small RNAs that bind to and inhibit the activity of CsrA (Lenz et al., 2005; Liu et al., 1997; Weilbacher et al., 2003). CsrA is a post-transcriptional regulator that can act as both an activator and a repressor. It is important for a number of regulatory functions, including carbohydrate metabolism and motility (Liu et al., 1995; Romeo & Gong, 1993; Romeo et al., 1993; Sabnis et al., 1995; Wei et al., 2001). In V. cholerae, VarS and VarA have been shown to regulate HapR expression levels by feeding into the quorum-sensing system at LuxO, so that varA mutants are deficient in HapR expression (Lenz et al., 2005). Here, we show that in addition to this mechanism of regulation, VarS and VarA also regulate the HapR regulon by altering HapR activity (Fig. 1).

**METHODS**

**Bacterial strains, plasmids and culture conditions.** All V. cholerae strains used in this study were derived from El Tor C6706 and are listed in Table 1. Except for when the luxCDABE<sub>V.h.</sub> reporter was used, strains were grown in Luria broth (LB) with the appropriate antibiotics at 37 °C under shaking conditions. Use of the luxCDABE<sub>V.h.</sub> reporter required growth at 30 °C. In-frame deletion mutants of luxO, hapR and varA were constructed as previously described (Lenz et al., 2005; Zhu et al., 2002). The in-frame deletion mutant for pckA and ve0191 was constructed by using pWM91 (Metcalf et al., 1996). The P<sub>varA</sub>-hapR plasmid was constructed by PCR-amplifying the hapR coding sequence (beginning with the ATG start codon) and cloning into pBBR1-MCS2 (Kovach et al., 1995). The P<sub>BAD24</sub>-hapR plasmid was constructed by PCR-amplifying the hapR coding sequence and cloning into pBAD24 (Guzman et al., 1995). The P<sub>vca0880-luxCDABE</sub> plasmid was constructed as previously described (Tsou et al., 2009). The P<sub>lac</sub>-csrB plasmid was constructed by PCR-amplifying the intergenic region containing csrB and cloning into pBluescript II (SK+) (Stratagene). The P<sub>hapA-lacZ</sub> plasmid was constructed by PCR-amplifying the hapA coding sequence and cloning into pAH6 (Hsiao et al., 2006).

**Transposon screens.** A mariner transposon, pSC137 (Chiang & Mekalanos, 1999), was introduced onto the chromosome of each of the indicated V. cholerae parent strains, and transconjugants were selected on LB plates containing the appropriate antibiotics. Transconjugants with the desired luminescence and protease phenotypes were identified, and arbitrary PCR and sequencing were used to determine the transposon insertion sites.

**Luminescence assays.** Strains containing luxCDABE<sub>V.h.</sub> or P<sub>vca0880-luxCDABE</sub> were grown at 30 or 37 °C under shaking conditions to the indicated cell densities, and luminescence was measured by using a Bio-Tek Synergy HT spectrophotometer. Results are reported as light units normalized to OD<sub>600</sub>.

**Western production assays.** Liquid cultures of each of the indicated strains were grown in LB, and samples were collected at the desired time points. Cultures were normalized to OD<sub>600</sub> and lysed, and proteins were fractionated by size via SDS-PAGE. Proteins were then transferred from the gel to a nitrocellulose membrane and immunoblotted by using polyclonal rabbit anti-<i>Δ</i>-HapR antibody.

**RESULTS**

**The VarS/VarA two-component system is necessary for full HapR activity**

To identify potential additional factors that are necessary for HapR function, we conducted a transposon mutagenesis screen in a strain of V. cholerae with the endogenous copy of hapR deleted from the chromosome and with a
than the regulator and lies downstream of VarS, so while there may
also examined protease production, which is positively
phenotype as the transposon insertion mutant (Fig. 2b). We
performed a Western blot to determine HapR protein
expression levels in a varA deletion mutant complemented with
Plac-hapR, and used wild-type and a luxO deletion mutant as controls. In the wild-type strain, quorum sensing
regulation resulted in expression of HapR only at high cell
density, and in the luxO mutant, disruption of the
quorum-sensing pathway resulted in constitutive expres-
sion of HapR at both high and low cell densities. The hapR
deletion mutant complemented with Plac-hapR behaved
similarly to the quorum sensing-deficient luxO mutant and expressed HapR at both high and low cell densities
(Fig. 3a). Thus, HapR expression is independent of
quorum sensing regulation when it is expressed from the
Plac promoter, and should not be regulated by VarS and
VarA if their only input is through LuxO.

To determine whether VarA affects Plac-hapR expression
levels independently of LuxO, a Western blot analysis was
performed on a varA deletion mutant. As a control, we
showed that the hapR mutant did not express HapR protein,
but when it was complemented with Plac-hapR, HapR was expressed and was able to activate the
luxCDABEV.h. reporter (Fig. 3b). In the varA deletion
mutant, we did not detect HapR protein (Fig. 3b), which is
consistent with the previous study showing regulation by

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
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<tbody>
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<td>C6706 (pBB1)</td>
<td>Wild-type, (luxCDABEV.h.)</td>
<td>Miller et al. (2002)</td>
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<tr>
<td>MM307 (pBB1)</td>
<td>luxO deletion, (luxCDABEV.h.)</td>
<td>Miller et al. (2002)</td>
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<tr>
<td>MM194 (pBB1)</td>
<td>hapR deletion, (luxCDABEV.h.)</td>
<td>Miller et al. (2002)</td>
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<td>ACV8</td>
<td>hapR deletion, (Plac-hapR), (luxCDABEV.h.)</td>
<td>This study</td>
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<tr>
<td>ATV15-13</td>
<td>varA::Tn, hapR deletion, (P lac-hapR), (luxCDABEV.h.)</td>
<td>This study</td>
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<tr>
<td>ATV15-24</td>
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<td>ATE24</td>
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Table 1. V. cholerae and E. coli strains used in this study

constitutively expressed copy that was no longer under
quorum-sensing control encoded on a plasmid. The
heterologous luxCDABE operon from V. harveyi
(luxCDABEV.h.) and the endogenous hapA were used to
measure HapR activity. An initial non-saturating screen of
approximately 10,000 mutants revealed that transposon
insertions in either varS or varA resulted in decreased HapR
activity (Fig. 2a). The varA mutant had a stronger phenotype
than the varS mutant, which was observed in a previous
study, and this may be because VarA is the response
regulator and lies downstream of VarS, so while there may
be other inputs into VarA that are still present in the varS
mutant, all signalling through the two-component system is
abolished when varA is deleted (Lenz et al., 2005). To verify
the results of the mutagenesis screen, we tested an in-frame
varA deletion mutant and found that it had the same
phenotype as the transposon insertion mutant (Fig. 2b). We
also examined protease production, which is positively
regulated by the quorum sensing regulator HapR (Jobling &
Holmes, 1997), in these mutants. Similar to what is seen
with the lux reporter, varA and varS mutants did not
produce proteases when assayed on milk agar plates (Fig. 2a,
b, lower panels). As we obtained both VarA and VarS from
our screen, we thought it was likely that signalling through
this two-component system would be important in the
regulation of HapR activity and decided to investigate the
mechanism of this regulation.

VarA affects HapR activity

The VarS/VarA two-component signalling system had
previously been shown to feed into the V. cholerae
quorum-sensing pathway through LuxO and therefore to
regulate HapR expression post-transcriptionally via the
quorum-sensing regulatory small RNAs (Lenz et al., 2005).
However, in the strain used for our screen, HapR is
expressed constitutively from the Plac promoter, and the
binding site for the quorum sensing-regulated small RNAs
is no longer present, so HapR expression should not be
under quorum-sensing control. To confirm this, we
performed a Western blot to determine HapR protein
expression expression levels in a hapR deletion mutant complemented with
P lac-hapR, and used wild-type and a luxO deletion mutant as controls. In the wild-type strain, quorum sensing
regulation resulted in expression of HapR only at high cell
density, and in the luxO mutant, disruption of the
quorum-sensing pathway resulted in constitutive expres-
sion of HapR at both high and low cell densities. The hapR
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similarly to the quorum sensing-deficient luxO mutant and expressed HapR at both high and low cell densities
(Fig. 3a). Thus, HapR expression is independent of
quorum sensing regulation when it is expressed from the
Plac promoter, and should not be regulated by VarS and
VarA if their only input is through LuxO.

To determine whether VarA affects P lac-hapR expression
levels independently of LuxO, a Western blot analysis was
performed on a varA deletion mutant. As a control, we
showed that the hapR mutant did not express HapR protein,
but when it was complemented with P lac-hapR, HapR was expressed and was able to activate the
luxCDABEV.h. reporter (Fig. 3b). In the varA deletion
mutant, we did not detect HapR protein (Fig. 3b), which is
consistent with the previous study showing regulation by
VarA of HapR protein levels through LuxO (Lenz et al., 2005). However, when the varA mutant was complemented with P_tac-hapR, even though HapR was expressed at a level comparable with what was seen with complementation of the hapR mutant, the level of activation of the luxCDABE_{V.h.} reporter was approximately 30-fold lower.

**Fig. 2.** Mutations in the VarS/VarA two-component system affect HapR activity. Transposon insertion (a) and in-frame deletion (b) mutants of varS or varA with constitutively expressed HapR were assayed for their ability to activate light production by the heterologous luxCDABE operon from V. harveyi and protease production by endogenous hapA. Luminescence data are presented as the mean of three experiments; error bars, SD. Protease activity was assayed by examining the extent of clearing on milk plates. Milk plates were made by adding 1% powdered milk to LB agar with the appropriate antibiotics.

VarA affects HapR activity without affecting protein levels when HapR is expressed from the P_tac promoter. (a) Western blot analysis for HapR protein levels was performed on the indicated strains at both high (OD_{600}=0.75) and low (OD_{600}=0.3) cell densities; WT, wild type. (b) Western blot analysis for HapR protein levels was performed for the indicated strains. At the time of sample collection, luminescence activity was also measured. (c) Luminescence was measured for the indicated strains after growth with the indicated concentrations of arabinose (Ara). The luminescence data are presented as the mean of three samples; error bars, SD. One representative sample for each strain and arabinose concentration was collected at the time at which luminescence was measured and was used for Western blot analysis of HapR protein levels.

**Fig. 3.**
We then performed essentially the same experiment using a different promoter to drive HapR expression and a different reporter to assess HapR activity to make sure that our observations were not specific to the promoter or reporter. In this new strain, arabinose was used to induce expression of HapR from the P_{BAD} promoter. The vca0880 promoter, which had previously been shown to be strongly activated by HapR, was used as the reporter (Tsou et al., 2009). Our results with the new promoter and reporter supported our finding that VarA affects HapR activity without altering HapR protein levels when HapR expression is no longer under quorum-sensing control (Fig. 3c).

Supplementation of the hapR deletion mutant with arabinose-induced HapR expressed from the P_{BAD} promoter resulted in HapR protein expression and activation of the target promoter. On the other hand, even though complementation of the varA hapR double mutant with HapR expressed from the same promoter with the same arabinose concentration resulted in approximately the same amount of HapR protein, HapR was unable to activate transcription from the vca0880 promoter without VarA. Thus, our results indicate that VarA modulates the ability of HapR to regulate its target genes independently of its previously described role in regulating HapR levels post-transcriptionally through the quorum-sensing system. This is consistent with the previous study in which regulation by VarA of HapR through the quorum-sensing system was first described (Lenz et al., 2005), because it was observed then that P_{lac} had not yet been shown to be able to fully complement a varA mutant in terms of HapR activation of luxCDABE_{V.ch} whereas it is able to fully complement a hapA mutant. However, the deficiency was not as drastic as that seen in the present study, perhaps due to differences in the constructs resulting in different levels of HapR expression (Lenz et al., 2005).

The effect of CsrB on HapR activity is independent of the quorum-sensing pathway

As two-component systems generally regulate many downstream targets, we thought it was likely that VarA could affect HapR expression (via the quorum-sensing pathway) through CsrB, but affect HapR activity through a distinct pathway that is independent of CsrB. However, our transposon mutagenesis screen and follow-up studies indicated that the effect of VarA on HapR activity was also through CsrB. CsrB is a small RNA that titrates the global regulator CsrA away from its target mRNA transcripts (Liu et al., 1997). As CsrB has been shown to act through LuxO to regulate HapR expression levels, we tested whether the Var/Csr system required LuxO to regulate HapR activity as well. We chose to do this by performing experiments in the MC4100 strain of E. coli, as E. coli does not contain LuxO or any of the other components of the central V. cholerae quorum-sensing pathway, but does contain CsrA. By using this experimental system, we could observe Csr regulation of HapR that was independent of the rest of the V. cholerae quorum-sensing pathway. HapR was able to activate transcription from the luxCDABE_{V.ch} and hapA promoters in E. coli without the addition of any other V. cholerae-specific factors (Fig. 5).

We wanted to see whether expressing csrB could further increase activity of HapR at these two promoters. CsrB is able to increase HapR-induced transcription from the luxCDABE_{V.ch} (Fig. 5a) and hapA promoters (Fig. 5b) in E. coli. Importantly, CsrB did not increase transcription from the luxCDABE_{V.ch} or hapA promoters in the absence of HapR (data not shown). Therefore, we concluded that VarA activates CsrB expression to regulate a downstream pathway other than the quorum-sensing system, and that...
this other pathway is important for the function of HapR as a transcriptional activator. This is consistent with the reported regulation by VarA of HapR levels through the quorum-sensing system (Lenz et al., 2005), because both the varA luxO and the varS luxO double mutants analysed in that study were constitutively bright, indicating that VarA and VarS do act through LuxO, although both double mutants were significantly darker than the luxO single mutant, suggesting that VarA and VarS also have effects on HapR activity independent of LuxO.

CsrB acts through PckA to modulate HapR activity

Once we had confirmed that CsrB does not act through the known V. cholerae quorum-sensing pathway to regulate HapR activity, we performed a transposon mutagenesis screen to identify the intermediate factor(s) linking CsrB to HapR activity. After transposon mutagenesis of hapR varA (Ptac-hapR) (Ptac-csrB) (luxCDABEh.vh), we looked for resulting mutants that were no longer able to activate protease or light production. We had two hits in pckA (Fig. 6a), whose expression is known to be repressed by CsrA (Romeo et al., 1993). As CsrB normally represses CsrA, which represses pckA, PckA is expressed at high levels when CsrB is expressed. Deleting pckA in a strain that constitutively expressed CsrB was sufficient to lower HapR activity significantly, both at the luxCDABEh.vh promoter (Fig. 6b) and at the hapA promoter (data not shown), suggesting that PckA is crucial for the influence of CsrB on HapR activity. The phosphoenolpyruvate carboxykinase encoded by pckA plays an important role in central carbon metabolism, specifically in gluconeogenesis. PckA may affect HapR activity by regulating the level of a carbon-metabolism intermediate that binds to and induces a conformational change in HapR, thus altering the ability of HapR to bind to its target promoters. Another possibility is that PckA affects the expression of a second transcriptional regulator whose presence is necessary for the activity of HapR.
HapR at each of its target promoters. The exact mechanism of this regulation is currently under investigation.

**DISCUSSION**

Quorum sensing allows bacteria to alter their gene expression profiles globally to respond appropriately to changing environmental conditions. Autoinducers transmit information about cell density into the cell by binding to cognate sensor kinases that trigger downstream phosphorelay pathways. In *V. cholerae*, the quorum-sensing pathway regulates the expression level of the transcriptional regulator HapR, which then activates or represses the expression of many downstream targets important for virulence and environmental survival. HapR expression is tightly regulated by the quorum-sensing pathway via four small regulatory RNAs (Lenz et al., 2004). Regulatory loops exist to precisely modulate the kinetics of quorum sensing regulation as *V. cholerae* shifts between group and individual behaviours (Svenningsen et al., 2008). This sophisticated cell density-dependent regulatory network is also integrated with other sensory networks to further tailor gene expression profiles to specific situations. For example, when *V. cholerae* penetrates the mucosal layer overlying the intestinal epithelium during colonization, its flagella break off, resulting in an increase in FliA levels. FliA represses HapR expression, and thus the motility pathway works in conjunction with the quorum-sensing pathway to maximally repress HapR expression during the initial stages of colonization, when cell density is low. This repression of HapR allows for maximal expression of virulence genes early in the infectious cycle (Liu et al., 2008). Additionally, HapR is regulated by VqmA, which is thought to respond to an as-yet-unidentified environmental signal (Liu et al., 2006). The VarS/VarA two-component system has previously been shown to regulate HapR expression in parallel with the central quorum-sensing pathway, with convergence at the level of LuxO (Lenz et al., 2005). Here, we show that in addition to this mechanism of altering HapR expression levels, VarA also regulates quorum-sensing targets by modulating HapR activity. Interestingly, we previously demonstrated that HapR represses transcription from the varA promoter (Tsou et al., 2009). Thus, it is clear that the VarS/VarA system and the quorum-sensing system are closely related, and that each is able to provide input into as well as receive feedback from the other system.

In this work, we demonstrate that a downstream target of VarA is important for HapR activity at the luxCDABE*<sub>v.h.</sub>*, hapA and vca0880 promoters. We further show that this downstream target is regulated by VarA through the small RNA CsrB, but acts independently of the central *V. cholerae* quorum-sensing system to influence HapR activity. As this target is necessary for activity at all of the HapR-regulated promoters that were tested, we believe it is most likely that it somehow modulates HapR activity. However, it remains possible that this target acts at each promoter independently of HapR and that both this target and HapR are necessary for full activation of transcription from these promoters. Finally, we show that the VarA target that modulates HapR activity probably does so through PckA. PckA is a key player in central carbon metabolism and, as such, affects the levels of many metabolic intermediates. Given that crystallization of HapR has revealed a potential ligand-binding pocket (De Silva et al., 2007), it is intriguing to think that one of these metabolic intermediates may bind to the ligand-binding pocket, induce a conformational change and affect HapR binding to target promoters.

As a global regulator of carbon metabolism, CsrA activates the expression of glycolytic genes and represses genes important for gluconeogenesis (Sabnis et al., 1995). One study has found that the presence of the BarA/UvrY system in *E. coli* allows for efficient switching between different carbon sources (Pernestig et al., 2003). Presumably,
efficient utilization of available carbon sources is crucial for survival in both environmental reservoirs and host organisms. Since the VarA/VarS system affects the quorum-sensing system regulatory transcription factor HapR at two levels (protein expression and activity), these two systems appear to be intricately intertwined. It is interesting to think that perhaps this is a way for \textit{V. cholerae} to combine information about surrounding cell density and nutrient availability to adjust its gene expression profile more precisely to reflect its surrounding conditions.

**ACKNOWLEDGEMENTS**

We thank the members of our laboratory for technical support. This study was supported by the National Institutes of Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) R01 (AI072479) (to J.Z.), and an NIH T32 EID training grant (to A.M.T.).

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Edited by: T. Msadek