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

Amy M. Tsou, Zhi Liu, Tao Cai and Jun Zhu

Correspondence

Jun Zhu

junzhu@mail.med.upenn.edu

Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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.

Received 18 October 2010

Revised 3 March 2011

Accepted 4 March 2011

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 phosphorylates 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*,

Abbreviation: Cm^R, chloramphenicol resistance.

Fig. 1. Model of the interactions between the *V. cholerae* quorum-sensing network and the VarS/VarA pathway. The previously described pathway is represented by the dotted arrow and the pathway described in this study is depicted with a dashed arrow; qrr sRNAs, quorum-regulatory small regulatory RNAs.

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; Weillbacher *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_{V.h.}* reporter was used, strains were grown in Luria broth (LB) with the appropriate antibiotics at 37 °C under shaking conditions. Use of the *luxCDABE_{V.h.}* 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 *vc0191* was constructed by using pWM91 (Metcalfe *et al.*, 1996). The *P_{lac}-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_{BAD}-hapR* plasmid was constructed by PCR-amplifying the *hapR* coding sequence and cloning into pBAD24 (Guzman *et al.*, 1995). The *P_{vca0880}-luxCDABE* plasmid was constructed as previously described (Tsou *et al.*, 2009). The *P_{lac}-csrB* plasmid was constructed by PCR-amplifying the intergenic region containing *csrB* and cloning into pBluescript II (SK+) (Stratagene). The *P_{hapA}-lacZ* 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_{V.h.}* or *P_{vca0880}-luxCDABE* 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 OD₆₀₀.

Western blotting for HapR. The indicated *V. cholerae* strains were grown in LB, and samples were collected at the desired time points. Cultures were normalized to OD₆₀₀ 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 (α)-HapR antibody.

Protease production assays. Liquid cultures of each of the indicated strains were grown to comparable cell densities, and equal volumes of each strain were spotted onto LB plates containing 1 % milk and the appropriate antibiotics. The plates were incubated at 37 °C overnight, and the extent of clearing around each spot was noted the following day.

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

Table 1. *V. cholerae* and *E. coli* strains used in this study

Strain	Genotype	Reference or source
<i>V. cholerae</i> (El Tor) strains		
C6706 (pBB1)	Wild-type, (<i>luxCDABE_{V.h.}</i>)	Miller <i>et al.</i> (2002)
MM307 (pBB1)	<i>luxO</i> deletion, (<i>luxCDABE_{V.h.}</i>)	Miller <i>et al.</i> (2002)
MM194 (pBB1)	<i>hapR</i> deletion, (<i>luxCDABE_{V.h.}</i>)	Miller <i>et al.</i> (2002)
ACV8	<i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)	This study
ATV15-13	<i>varA::Tn</i> , <i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)	This study
ATV15-24	<i>varS::Tn</i> , <i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)	This study
MM560	<i>varA</i> deletion, (<i>luxCDABE_{V.h.}</i>)	Lenz <i>et al.</i> (2005)
ATV18	<i>varA</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)	This study
ATV19	<i>varA</i> deletion, <i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)	This study
ATV20	<i>hapR</i> deletion, (<i>P_{BAD}-hapR</i>), (<i>P_{vca0880}-lux</i>)	This study
ATV21	<i>varA</i> deletion, <i>hapR</i> deletion, (<i>P_{BAD}-hapR</i>), (<i>P_{vca0880}-lux</i>)	This study
ATV22	<i>varA</i> deletion, <i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)(vector)	This study
ATV23	<i>varA</i> deletion, <i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>)(<i>P_{lac}-csrB</i>)	This study
ATV17-21 and ATV 17-39	<i>pckA::Tn</i> , <i>varA</i> deletion, <i>hapR</i> deletion, (<i>P_{tac}-hapR</i>), (<i>P_{lac}-csrB</i>), (<i>luxCDABE_{V.h.}</i>)	This study
ATV24	<i>pckA</i> deletion, (<i>luxCDABE_{V.h.}</i>)	This study
<i>E. coli</i> (MC4100) strains		
ATE21	(<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>), (vector)	This study
ATE22	(<i>P_{tac}-hapR</i>), (<i>luxCDABE_{V.h.}</i>), (<i>P_{lac}-csrB</i>)	This study
ATE23	(<i>P_{tac}-hapR</i>), (<i>P_{hapA}-lacZ</i>), (vector)	This study
ATE24	(<i>P_{tac}-hapR</i>), (<i>P_{hapA}-lacZ</i>), (<i>P_{lac}-csrB</i>)	This study

constitutively expressed copy that was no longer under quorum-sensing control encoded on a plasmid. The heterologous *luxCDABE* operon from *V. harveyi* (*luxCDABE_{V.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 *P_{tac}* 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 levels in a *hapR* deletion mutant complemented with *P_{tac}-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 expression of HapR at both high and low cell densities. The *hapR* deletion mutant complemented with *P_{tac}-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 *P_{tac}* promoter, and should not be regulated by VarS and VarA if their only input is through LuxO.

To determine whether VarA affects *P_{tac}-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_{tac}-hapR*, HapR was expressed and was able to activate the *luxCDABE_{V.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

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 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. 3. 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. 3b). 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). Complementation 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_{tac} -*hapR* is not able to fully complement a *varA* mutant in terms of HapR activation of *luxCDABE_{V,h}*, whereas it is able to fully complement a *hapR* 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 influence of VarA on HapR activity is mediated through CsrB

To elucidate the mechanism by which VarA mediates its effect on HapR activity, we performed a transposon mutagenesis screen on the *varA hapR* strain that was complemented with P_{tac} -*hapR* and used *luxCDABE_{V,h}* as a reporter for HapR activity. Bright mutants that were able to bypass the *varA* deletion were identified, and the HapA protease activity for each mutant was assessed to eliminate those insertions that specifically altered the ability of the bacteria to produce light. More than 10 000 mutant colonies were screened, and insertions in one particular region consistently produced a strong luminescence and protease phenotype. All three of the insertions occurring here, which were found in different mutant pools, were located at the tail end of the *vc0191* locus. We constructed an in-frame deletion mutant of *vc0191* in the background of the parent strain for the screen to see if it was responsible for the observed phenotype. Deleting *vc0191* did not affect luminescence (data not shown), so we looked more closely at the three insertions to see how else they might be altering gene expression. The *vc0191* deletion

result was not entirely surprising because one of the transposon insertions actually occurred just after the end of the *vc0191* ORF. Additionally, the transposon used in the screen contained a chloramphenicol-resistance (Cm^R) gene for selection purposes, and all three of the insertions occurred such that the Cm^R promoter pointed in the same direction, into the intergenic region between *vc0190* and *vc0191* (Fig. 4a). The small RNA CsrB had previously been shown to be encoded in this intergenic region (Lenz *et al.*, 2005), in the same orientation as the Cm^R promoters, so we thought it was possible that the insertions were causing the observed phenotype by driving expression of CsrB. To determine if this was the case, we constitutively expressed CsrB from a plasmid under the control of the P_{lac} promoter and transformed that plasmid into the parent strain for the screen. Indeed, we observed that CsrB overexpression allowed bypass of the *varA* deletion so that HapR could activate transcription from the *luxCDABE_{V,h}*, as well as the *hapA* promoter (Fig. 4b). Since it is known that VarA activates the transcription of CsrB (Lenz *et al.*, 2005), these results indicate that VarA acts through CsrB to modulate HapR activity, and therefore in the absence of VarA, expression of CsrB can restore HapR activity.

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 CsrA 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,h}* 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,h}* (Fig. 5a) and *hapA* promoters (Fig. 5b) in *E. coli*. Importantly, CsrB did not increase transcription from the *luxCDABE_{V,h}* 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

Fig. 4. VarA acts through the small RNA CsrB to regulate HapR activity. (a) Transposon insertions that lead to overexpression of *csrB*. Arrows indicate the transcriptional orientation of the Cm^R gene in the transposon. (b) A *varA* mutant strain that expressed HapR constitutively was transformed with either a vector or a plasmid expressing CsrB, and the resulting strains were assayed for their abilities to activate light production by the heterologous *luxCDABE* operon from *V. harveyi* and protease activity by the endogenous *hapA*. Luminescence data are presented as the mean of three experiments; error bars, SD. Cultures were also spotted on plates, and photographs were taken after overnight incubation at 30 °C (lower panel). 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.

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

Fig. 5. Regulation of HapR activity by CsrB is independent of the central *V. cholerae* quorum-sensing system. (a) *E. coli* MC4100 strains containing $P_{tac}\text{-hapR}$ and *luxCDABE*_{*V.h.*} with either a vector control or $P_{lac}\text{-csrB}$ were assayed for light production. (b) *E. coli* MC4100 strains containing $P_{tac}\text{-hapR}$ and $P_{hapA}\text{-lacZ}$ with either a vector control or $P_{lac}\text{-csrB}$ were assayed for β -galactosidase activity. All experiments were performed in triplicate, and the data are presented as the mean of the three samples; error bars, SD.

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* ($P_{tac}\text{-hapR}$) ($P_{lac}\text{-csrB}$) (*luxCDABE*_{*V.h.*}), 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 *luxCDABE*_{*V.h.*} 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

Fig. 6. Regulation of HapR activity by CsrB is dependent upon PckA. (a) Liquid cultures of the indicated strains were grown to late-exponential phase, and equal volumes of each were then spotted onto plates containing the appropriate antibiotics with or without 1% milk. Plates without milk were incubated at 30 °C overnight, and the degree of light production was observed the following day. Plates with milk were incubated at 37 °C overnight, and the extent of protease-mediated clearing around each spot was noted the following day. (b) Light production by each of the indicated strains was measured and normalized by cell density. The data are reported as the mean of three experiments; error bars, SD.

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 FlhA levels. FlhA 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*_{V,h}, *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 *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/NIAD) R01 (AI072479) (to J.Z.), and an NIH T32 EID training grant (to A.M.T.).

REFERENCES

- Chiang, S. L. & Mekalanos, J. J. (1999). *rfb* mutations in *Vibrio cholerae* do not affect surface production of toxin-coregulated pili but still inhibit intestinal colonization. *Infect Immun* **67**, 976–980.
- De Silva, R. S., Kovackikova, G., Lin, W., Taylor, R. K., Skorupski, K. & Kull, F. J. (2007). Crystal structure of the *Vibrio cholerae* quorum-sensing regulatory protein HapR. *J Bacteriol* **189**, 5683–5691.
- Gao, R., Mack, T. R. & Stock, A. M. (2007). Bacterial response regulators: versatile regulatory strategies from common domains. *Trends Biochem Sci* **32**, 225–234.
- Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177**, 4121–4130.
- Hammer, B. K. & Bassler, B. L. (2003). Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol* **50**, 101–104.
- Heeb, S. & Haas, D. (2001). Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol Plant–Microbe Interact* **14**, 1351–1363.
- Herren, C. D., Mitra, A., Palaniyandi, S. K., Coleman, A., Elankumaran, S. & Mukhopadhyay, S. (2006). The BarA-UvrY two-component system regulates virulence in avian pathogenic *Escherichia coli* O78:K80:H9. *Infect Immun* **74**, 4900–4909.
- Hsiao, A., Liu, Z., Joelsson, A. & Zhu, J. (2006). *Vibrio cholerae* virulence regulator-coordinated evasion of host immunity. *Proc Natl Acad Sci U S A* **103**, 14542–14547.
- Jobling, M. G. & Holmes, R. K. (1997). Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol Microbiol* **26**, 1023–1034.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Lenz, D. H., Mok, K. C., Lilley, B. N., Kulkarni, R. V., Wingreen, N. S. & Bassler, B. L. (2004). The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118**, 69–82.
- Lenz, D. H., Miller, M. B., Zhu, J., Kulkarni, R. V. & Bassler, B. L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol* **58**, 1186–1202.
- Liu, M. Y., Yang, H. & Romeo, T. (1995). The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. *J Bacteriol* **177**, 2663–2672.
- Liu, M. Y., Gui, G., Wei, B., Preston, J. F., III, Oakford, L., Yüksel, U., Giedroc, D. P. & Romeo, T. (1997). The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J Biol Chem* **272**, 17502–17510.
- Liu, Z., Hsiao, A., Joelsson, A. & Zhu, J. (2006). The transcriptional regulator VqmA increases expression of the quorum-sensing activator HapR in *Vibrio cholerae*. *J Bacteriol* **188**, 2446–2453.
- Liu, Z., Miyashiro, T., Tsou, A., Hsiao, A., Goulian, M. & Zhu, J. (2008). Mucosal penetration primes *Vibrio cholerae* for host colonization by repressing quorum sensing. *Proc Natl Acad Sci U S A* **105**, 9769–9774.
- Metcalfe, W. W., Jiang, W., Daniels, L. L., Kim, S. K., Haldimann, A. & Wanner, B. L. (1996). Conditionally replicative and conjugative plasmids carrying *lacZ* for cloning, mutagenesis, and allele replacement in bacteria. *Plasmid* **35**, 1–13.
- Miller, M. B., Skorupski, K., Lenz, D. H., Taylor, R. K. & Bassler, B. L. (2002). Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* **110**, 303–314.
- Mitrophanov, A. Y. & Groisman, E. A. (2008). Signal integration in bacterial two-component regulatory systems. *Genes Dev* **22**, 2601–2611.
- Neiditch, M. B., Federle, M. J., Pompeani, A. J., Kelly, R. C., Swem, D. L., Jeffrey, P. D., Bassler, B. L. & Hughson, F. M. (2006). Ligand-induced asymmetry in histidine sensor kinase complex regulates quorum sensing. *Cell* **126**, 1095–1108.
- Pernestig, A. K., Melefors, O. & Georgellis, D. (2001). Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. *J Biol Chem* **276**, 225–231.
- Pernestig, A. K., Georgellis, D., Romeo, T., Suzuki, K., Tomenius, H., Normark, S. & Melefors, O. (2003). The *Escherichia coli* BarA-UvrY two-component system is needed for efficient switching between glycolytic and gluconeogenic carbon sources. *J Bacteriol* **185**, 843–853.
- Romeo, T. & Gong, M. (1993). Genetic and physical mapping of the regulatory gene *csrA* on the *Escherichia coli* K-12 chromosome. *J Bacteriol* **175**, 5740–5741.
- Romeo, T., Gong, M., Liu, M. Y. & Brun-Zinkernagel, A. M. (1993). Identification and molecular characterization of *csrA*, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J Bacteriol* **175**, 4744–4755.
- Sabnis, N. A., Yang, H. & Romeo, T. (1995). Pleiotropic regulation of central carbohydrate metabolism in *Escherichia coli* via the gene *csrA*. *J Biol Chem* **270**, 29096–29104.
- Svenningsen, S. L., Waters, C. M. & Bassler, B. L. (2008). A negative feedback loop involving small RNAs accelerates *Vibrio cholerae*'s transition out of quorum-sensing mode. *Genes Dev* **22**, 226–238.
- Tomenius, H., Pernestig, A. K., Jonas, K., Georgellis, D., Möllby, R., Normark, S. & Melefors, O. (2006). The *Escherichia coli* BarA-UvrY two-component system is a virulence determinant in the urinary tract. *BMC Microbiol* **6**, 27.
- Tsou, A. M., Cai, T., Liu, Z., Zhu, J. & Kulkarni, R. V. (2009). Regulatory targets of quorum sensing in *Vibrio cholerae*: evidence for two distinct HapR-binding motifs. *Nucleic Acids Res* **37**, 2747–2756.
- Wei, B. L., Brun-Zinkernagel, A. M., Simecka, J. W., Prüss, B. M., Babitzke, P. & Romeo, T. (2001). Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Mol Microbiol* **40**, 245–256.

Weilbacher, T., Suzuki, K., Dubey, A. K., Wang, X., Gudapaty, S., Morozov, I., Baker, C. S., Georgellis, D., Babitzke, P. & Romeo, T. (2003). A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. *Mol Microbiol* **48**, 657–670.

Wong, S. M., Carroll, P. A., Rahme, L. G., Ausubel, F. M. & Calderwood, S. B. (1998). Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. *Infect Immun* **66**, 5854–5861.

Zhu, J. & Mekalanos, J. J. (2003). Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell* **5**, 647–656.

Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L. & Mekalanos, J. J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **99**, 3129–3134.

Edited by: T. Msadek