

A simple mechanism for integration of quorum sensing and cAMP signalling in *V. cholerae*

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Abstract

Many bacteria use quorum sensing to control changes in lifestyle. The process is regulated by microbially derived “autoinducer” signalling molecules, that accumulate in the local environment. Individual cells sense autoinducer abundance, to infer population density, and alter their behaviour accordingly. In *Vibrio cholerae*, quorum sensing signals are transduced by phosphorelay to the transcription factor LuxO. Unphosphorylated LuxO permits expression of HapR, which alters global gene expression patterns. In this work, we have mapped the genome-wide distribution of LuxO and HapR in *V. cholerae*. Whilst LuxO has a small regulon, HapR targets 32 loci. Many HapR targets coincide with sites for the cAMP receptor protein (CRP) that regulates the transcriptional response to carbon starvation. This overlap, also evident in other *Vibrio* species, results from similarities in the DNA sequence bound by each factor. At shared sites, HapR and CRP simultaneously contact the double helix and binding is stabilised by direct interaction of the two factors. Importantly, this involves a CRP surface that usually contacts RNA polymerase to stimulate transcription. As a result, HapR can block transcription activation by CRP. Thus, by interacting at shared sites, HapR and CRP integrate information from quorum sensing and cAMP signalling to control gene expression. This likely allows *V. cholerae* to regulate subsets of genes during the transition between aquatic environments and the human host.

eLife assessment

This paper provides **valuable** new information on the mechanisms by which *Vibrio cholerae* integrates and responds to environmental signals. The strength of the evidence provided in support of the conclusions made and the model proposed is **solid**. The revision resolved many of the issues raised by the reviewers and improved the manuscript. The work is relevant for a broad audience of microbiologists interested in the mechanisms by which bacteria sense their environment.

Introduction

Vibrio cholerae is a Gram-negative bacterium responsible for the human disease cholera⁽¹⁾. Estimates suggest 3 million annual infections, of which 100 thousand are fatal⁽²⁾. Most disease instances are attributed to the El Tor *V. cholerae* biotype, which is responsible for the ongoing 7th cholera pandemic⁽³⁾. Globally, over 1 billion people inhabit areas of endemicity and future climatic change is likely to exacerbate the risk of illness^{(2), (4)}. The success of *V. cholerae* as a pathogen is underpinned by an ability to colonise both aquatic ecosystems and the human intestinal tract⁽¹⁾. In waterways, *V. cholerae* prospers by forming biofilms on arthropod exoskeletons. Degradation of these chitinous surfaces ultimately liberates *N*-acetylglucosamine (GlcNAc) for metabolism by the microbe⁽⁵⁾. Upon ingestion by a human host, *V. cholerae* express genetic determinants for acid tolerance, intestinal colonisation, and virulence. Diverse transcription factors regulate the transition and respond to signals including bile⁽⁶⁾, temperature⁽⁷⁾, nucleotide second messengers^{(8), (9)}, and chitin availability⁽⁵⁾. Understanding these regulatory networks is important to determine how *V. cholerae* can switch between environments to cause disease outbreaks^{(3), (10), (11)}.

Quorum sensing is key for the transition of *V. cholerae* between ecological niches⁽¹²⁾. Briefly, *V. cholerae* produce at least 3 autoinducer (AI) signalling molecules: cholera AI-1 (CAI-1), AI-2, and 3,5-dimethylpyrazin-2-ol (DPO)⁽¹³⁾. In the environment, these compounds are detected by receptors in neighbouring cells and indicate population density. Importantly, whilst AI-2 and DPO are produced by multiple bacterial species, CAI-1 is only made by other members of the *Vibrio* genus⁽¹⁴⁾. Thus, *V. cholerae* can determine the crude composition of bacterial populations. In the absence of their cognate AIs, when population density is low, the receptors for CAI-1 and AI-2 target the transcription factor LuxO for phosphorylation via a phosphorelay system^{(13), (15), (16)}. When phosphorylated, LuxO upregulates the production of four small quorum regulatory RNAs (Qrrs)⁽¹⁷⁾. In turn, the Qrrs control expression of two global transcription factors: AphA and HapR^{(17)–(19)}. Importantly, whilst AphA production is activated by Qrrs, synthesis of HapR is repressed. Hence, AphA and HapR control gene expression at low and high cell density respectively^{(13), (19)}. A simplified outline of the LuxO dependent regulatory pathway for HapR is illustrated in [Figure 1a](#).

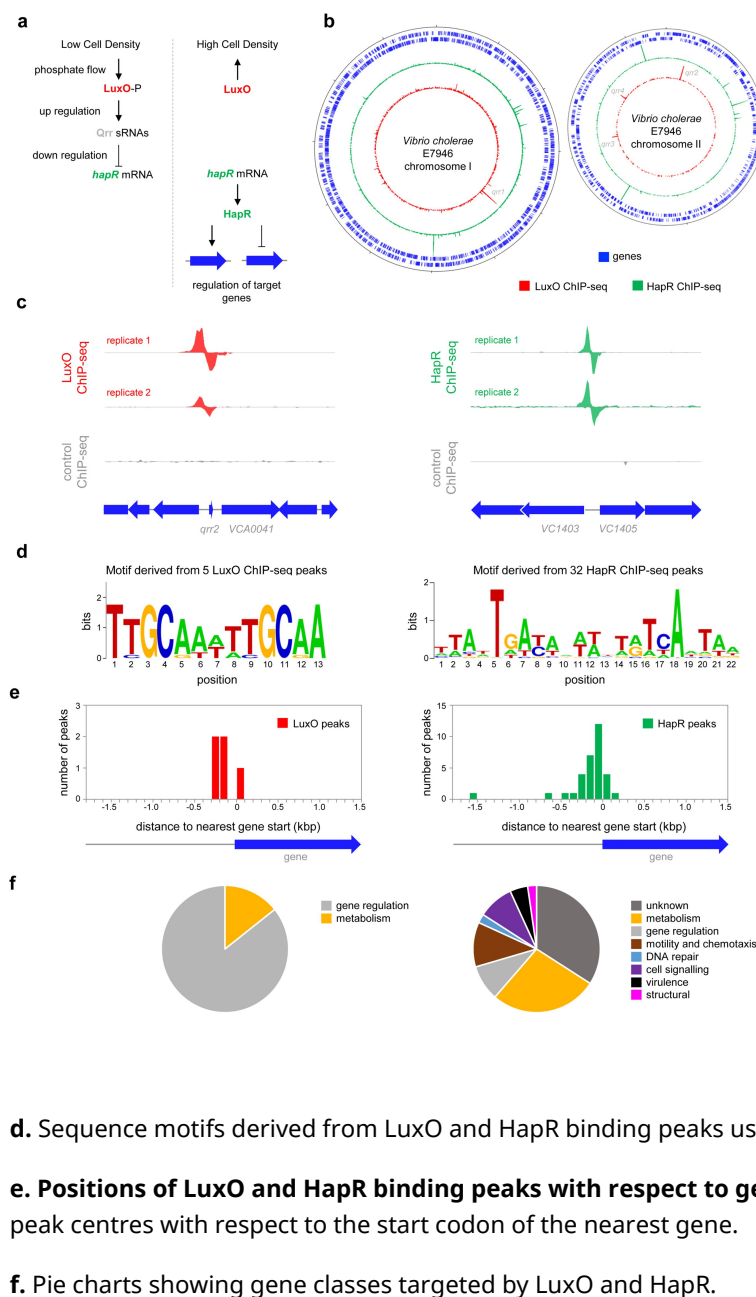


Figure 1

Genome-wide distribution of HapR and LuxO in *Vibrio cholerae*.

a. Simplified schematic overview of quorum sensing in *Vibrio cholerae*. At low cell density, expression of HapR is repressed by the Qrr sRNAs that depend on phosphorylated LuxO for activation of their transcription. Arrows indicate activation and bar ended lines indicate repression. For clarity, not all protein factors involved in the cascade have been included.

b. Binding of LuxO and HapR across both *Vibrio cholerae* chromosomes. In each plot the outer two tracks (blue) are genes orientated in the forward or reverse direction. The LuxO and HapR ChIP-seq binding signals are shown in red and green. LuxO binding peaks corresponding to the *qrr1-4* loci are indicated. Tick marks are 0.25 Mbp apart.

c. Example LuxO and HapR ChIP-seq binding peaks. ChIP-seq coverage plots are shown for individual experimental replicates. Data for LuxO and HapR are in green and red respectively. Signals above or below the horizontal line correspond to reads mapping to the top or bottom strand respectively. Gene are show as block arrows.

d. Sequence motifs derived from LuxO and HapR binding peaks using MEME.

e. Positions of LuxO and HapR binding peaks with respect to genes. The histograms show the distribution of binding peak centres with respect to the start codon of the nearest gene.

f. Pie charts showing gene classes targeted by LuxO and HapR.

Identified as a regulator of *hapA*, required for *V. cholerae* migration through intestinal mucosa, HapR is a TetR-family member that binds DNA as a homodimer via a N-terminal helix-turn-helix motif^{(20), (21)}. Many clinical isolates of pandemic *V. cholerae* have lost the ability to properly express HapR and this may indicate adaptation to a more pathogenic lifestyle^{(3), (10), (22)}. In *V. cholerae*, HapR regulates the expression of ~100 genes to promote 'group behaviours' including natural competence, repression of virulence genes, and escape from the host intestinal mucosa⁽²³⁾. In other *Vibrio* spp., equivalent regulons are larger. For example, LuxR in *Vibrio harveyi* regulates over 600 genes⁽²⁴⁾. Expression of HapR can be influenced by other factors. In particular, cAMP receptor protein (CRP), a regulator that controls metabolism of alternative carbon sources, including chitin, upregulates HapR⁽²⁵⁾. In this study, we used chromatin immunoprecipitation and DNA sequencing (ChIP-seq) to identify direct DNA binding targets of HapR and its upstream regulator, LuxO. We show that

the degenerate DNA consensus bound by HapR frequently overlaps targets for CRP. At such sites, HapR and CRP co-operatively bind offset faces of the double helix. Strikingly, this occludes a key CRP surface required to activate transcription. This simple mechanism allows *V. cholerae* species to integrate quorum sensing, and cAMP signalling, in the control of gene expression.

Results

Genome-wide DNA binding by HapR and LuxO in *Vibrio cholerae*

Whilst the impact of HapR on global gene expression in *V. cholerae* has been investigated, it is not known which HapR responsive genes are directly controlled by the protein⁽²³⁾. Similarly, the extent of the direct LuxO regulon is unknown. Hence, we sought to map the binding of LuxO and HapR across the *V. cholerae* genome. To facilitate this, *luxO* and *hapR* were cloned in plasmids pAMCF and pAMNF respectively. The resulting constructs, encoding LuxO-3xFLAG or 3xFLAG-HapR, were used to transform *V. cholerae* strain E7946. In subsequent ChIP-seq experiments, anti-FLAG antibodies were used to select fragments of the *V. cholerae* genome bound with either LuxO or HapR. The derived binding profiles are shown in [Figure 1b](#). In each plot, genes are shown as blue lines (outer two tracks) whilst the LuxO and HapR binding signals are red and green respectively (inner two tracks). Examples of individual binding peaks for each factor are shown in [Figure 1c](#). In total, we identified 5 and 32 peaks for LuxO and HapR binding respectively ([Table 1](#)). Previous work identified targets for LuxO adjacent to genes encoding the 4 Qrr sRNAs. We recovered all of these known LuxO targets, and an additional binding site was identified between *VC1142* and *VC1143*. These divergent genes encode cold shock-like protein CspD, and the Clp protease adaptor protein, ClpS, respectively. Note that the LuxO binding signal at this locus is small, compared to the *qrr1-4* targets, but may still be involved in transcription regulation ([Figure 1-figure supplement 1](#)). To identify the sequence bound by LuxO, DNA regions overlapping LuxO binding peaks were inspected using MEME. The motif identified matches the known consensus for LuxO binding and was found at all LuxO targets ([Table 1](#) and [Figure 1d](#))⁽²⁶⁾. The positions of LuxO binding sites with respect to genes, and the functions encoded by these genes, are summarised in [Figures 1e](#) and [1f](#) respectively.

Table 1:

Locations of binding peaks from ChIP-seq experiments

peak centre	gene(s) ⁴	site location	site sequence	TSS ⁵
HapR ChIP-seq peaks				
<i>chromosome I</i>				
99874	<i>VC0102</i> <(<i>VC0103</i>)	99863.5	aaattaataaaaactgtcattta	99906 (+)
213457	(<i>VC0205</i>)> <i>VC0206</i>	213452.5	taattgtgattccttatcaccaa	213494 (+)
246366 ¹	<i>VC0240</i> <> <i>VC0241</i>	246349.5	taattaagatggctataaacta	246430 (-)
463584	<i>VC0433</i>			
514422	<i>VC0484</i>	514430.5	ctactgaccttttcatcaataa	514427 (+)
516570	(<i>VC0486</i>)	516601.5	caactgagaaggcacacaatag	516545 (+)
534714	(<i>VC0502</i>)	534691.5	ctattataagctctatcagtg	534805 (-)
547108	<i>VC0515</i>	547135.5	atagtaataattattgttaata	549431 (-)
613328 ³	<i>VC0583</i> ^A	613357.5	ttattgagtggttacataacaa	613427 (+)
716707	<i>VC0668</i>	716625.5	ctattgatgaggttatccacag	716537 (-)
735309	<i>VC0687</i> <> <i>VC0688</i>			
882854	(<i>VC0822</i>)	882825.5	taattatccacttttatcaattg	883072 (-)
941187	<i>VC0880</i>	941164.5	cttttgacatttctgtcacaaa	941152 (+)
978577	<i>VC0916</i> ^R	978540.5	taattaatatccagctcaatta	978581 (+)
1356743	<i>VC1280</i> <> <i>VC1281</i> ^A	1356736.5	atattgatagaaataacaagtc	1356896 (+)
1379202	<i>VC1298</i> <> <i>VC1299</i>	1379180.5	ttcatgatagttttgttaattat	1379189 (+)
1469384	<i>VC1375</i> <> <i>VC1376</i>	1469377.5	atattgatataatcacacatctt	1469374 (+)
1496023	<i>VC1403</i> ^A <> <i>VC1405</i>	1496025.5	tagttgatatttttataaattgt	1495942 (+)
1533842	(<i>VC1437</i>)	1533854.5	tttgtagtctcctgtcaataa	1533703 (-)
1990133 ²	<i>VC1851</i>	1990076.5	atattgagtaatacaattagtaa	1990031 (+)
2364721	(<i>VC2212</i>)	2364680.5	ctattaacagttttatttataa	2364774 (+)
2509878	<i>VC2352</i>	2509882.5	ttagtgcagatgcgtcattaa	2509790 (-)
2667349	<i>VC2486</i>	2667368.5	taattattaatttgaacaatag	2667206 (-)
<i>chromosome II</i>				
163808 ¹	<i>VCA0148</i>	163810.5	taattgattattgtgtaactat	163852 (-)
214589	(<i>VCA0198</i>)	214582.5	taattgataactttgacagtat	213494 (+)
237008	<i>VCA0218</i> <> <i>VCA0219</i> ^R	237019.5	taaataatatgaatatcagtaa	237053 (+)
247286	<i>VCA0224</i> <> <i>VCA0225</i>	247241.5	taaatagactaataagacaatta	247165 (-)
598444	<i>VCA0662</i> <> <i>VCA0663</i> ^R	598403.5	tttgtaataaatttgtcattaa	598413 (+)
630517	<i>VCA0691</i> ^A	630559.5	ctattaacaggactgcattaa	631303 (+)
862737	<i>VCA0906</i>			
910196	<i>VCA0960</i> ^R <> <i>VCA0961</i>	910181.5	ctgattataaatttgttaaatat	910330 (+)
1021174	<i>VCA1070</i>	1021117.5	ctcctatccgattgggtcactat	1021326 (+)
LuxO ChIP-seq peaks				
<i>chromosome I</i>				
1090129	<i>qrr1</i> <> <i>VC1021</i>	1090154	ttgcaaaatgcaa	1090182 (+)
1212442	<i>VC1142</i> <> <i>VC1143</i>	1212435	ttgcaaatcgca	1212403 (-)
<i>chromosome II</i>				
48415	<i>qrr2</i>	48347	ttgcaatttgcaa	48851 (-)
772208	<i>qrr3</i>	772149	ttgcattttgcaa	772227 (+)
908445	<i>qrr4</i>	908436	ttgcaatttgcaa	908475 (+)

¹Identified by Tsou and co-workers²⁸

²Identified by Waters and co-workers²⁹

³Identified by Lin and co-workers²⁷

⁴Identified as activated (A) or repressed (R) by Nielsen and co-workers²³, VC0206, VC0240, VC0241, VC0583, VC0668, VC0916, VC1021, VC1142, VC1143, VCA0219 correspond to *murQ*, *rfaD*, *rfaA*, *hapR*, *mutH*, *vpsU*, *luxO*, *cspD*, *clpS*, *hlyA* respectively.

⁵Nearest transcription start site (TSS) identified by Papenfort *et al.*³² with the symbol in parenthesis indicating the direction of transcription. Note that these are not necessarily those TSSs subject to regulation by HarR or LuxO, particularly if the regulators are acting as repressors, or if the gene subject to regulation is switched off for another reason in the conditions of Papenfort *et al.*

Of the 32 peaks for HapR binding, 4 correspond to previously identified direct targets (*hapR*⁽²⁷⁾, *VC0241*⁽²⁸⁾, *VC1851*⁽²⁹⁾ and *VCA0148*⁽²⁸⁾). However, some known targets had weak or poorly reproducible binding signals (Figure 1-figure supplement 2). A DNA motif common

to all 32 HapR ChIP-seq peaks matched prior descriptions of the DNA target for HapR or closely related proteins^{(27), (30), (31)} (Figure 1d). Occurrences of this HapR binding motif were most frequent in the 200 bp preceding a gene start codon (Figure 1e). Most often, the genes adjacent to HapR binding peaks encode protein functions related to metabolism, motility, and chemotaxis (Figure 1f). Overall, our data suggest that LuxO primarily regulates gene expression via the 4 Qrr sRNA molecules. Conversely, the genome-wide distribution of HapR is consistent with that of a global gene regulator with many undefined regulatory roles.

HapR is a direct regulator of transcription at many target sites

We focused our attention on new HapR target promoters where adjacent coding sequence could be used to predict encoded protein function. For these 24 targets, regulatory DNA was cloned upstream of *lacZ* in plasmid pRW50T. Recombinants were then transferred to *V. cholerae* E7946, or the $\Delta hapR$ derivative, by conjugation. Strains generated were cultured overnight before β -galactosidase activities were determined. The results are shown in Figure 2a. Promoters were categorised as inactive, unresponsive, repressed or activated by HapR. We identified 2 and 7 promoters subject to activation and repression by HapR respectively. Of the remaining promoters, 6 were inactive and 9 unresponsive to HapR in our conditions. Next, the 9 promoter DNA fragments responsive to HapR *in vivo* were cloned upstream of the λoop terminator in plasmid pSR. The resulting constructs were then provided to housekeeping *V. cholerae* RNA polymerase, as templates for *in vitro* transcription, in the presence and absence of HapR. The results are shown in Figure 2b where the expected size of transcripts terminated by λoop are marked with blue triangles⁽³²⁾. Recall that the VC1375 and VC1403 promoters were activated by HapR *in vivo* (Figure 2a). Consistent with this, HapR also activated the VC1375 promoter *in vitro* (Figure 3b, lanes 43-47). However, HapR did not activate *in vitro* transcription from the VC1403 promoter (Figure 3b, lanes 48-53). Indeed, interpretation of these data were hampered because the location of the VC1403 transcription start site (TSS) is not known⁽³²⁾. Of the 7 promoters repressed by HapR *in vivo*, we observed repression in 6 cases *in vitro* (*hapR*, VC0585, VC2352, VCA0219, VCA0663 and VCA0960) (lanes 7-42). Conversely, the *murQP* promoter (*PmurQP*) subject to repression by HapR *in vivo*, generated no transcript *in vitro* (lanes 1-6). Full gel images are shown in Figure 2-figure supplement 1.

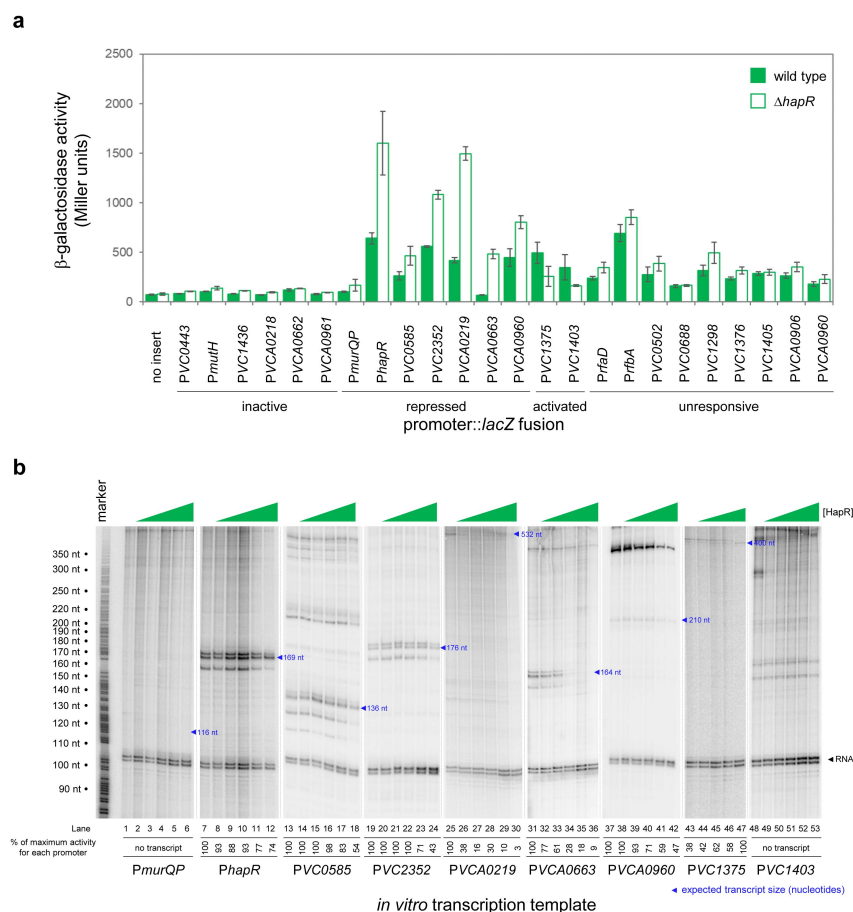


Figure 2:

HapR is a direct repressor of transcription at many target promoters.

a. Activity of HapR targeted promoters in the presence and absence of HapR *in vivo*. The promoter regions of HapR targeted genes were fused to *lacZ* in plasmid pRW50T and constructs used to transform required bacterial strains. β -galactosidase activity was measured in cell lysates taken from *Vibrio cholerae* E7946 (bars) or the Δ hapR derivative (open bars). containing the VC0857 promoter cloned upstream of *lacZ*. Standard deviation is shown for three independent biological replicates. Cells were grown in LB-Lennox medium at 37 °C to an OD₆₅₀ of ~1.1. Promoters were classified as inactive if, in both the presence and absence of HapR, β -galactosidase activity was <2-fold higher than the equivalent no insert control. We have labelled promoters with gene names or locus tags as most appropriate. Note that the Table 1 footnote can be used to cross

reference between locus tags and gene names where relevant.

b. Activity of HapR targeted promoters in the presence and absence of HapR *in vitro*. The gel images show results of *in vitro* transcription experiments. The DNA templates were plasmid pSR derivatives containing the indicated regulatory regions. Experiments were done with 0.4 μ M RNA polymerase in the presence (0.25, 0.75, 1.0, 3.0, or 5.0 μ M) and absence of HapR. Except for the VC1375 promoter, where the maximum HapR concentration was 3.0 μ M. The RNAI transcript is plasmid-derived and acts as an internal control. Expected transcript sizes, based on results from global transcription start site mapping experiments⁽³²⁾, are indicated. Note that no VC1403 transcript was detected in this prior study⁽³²⁾.

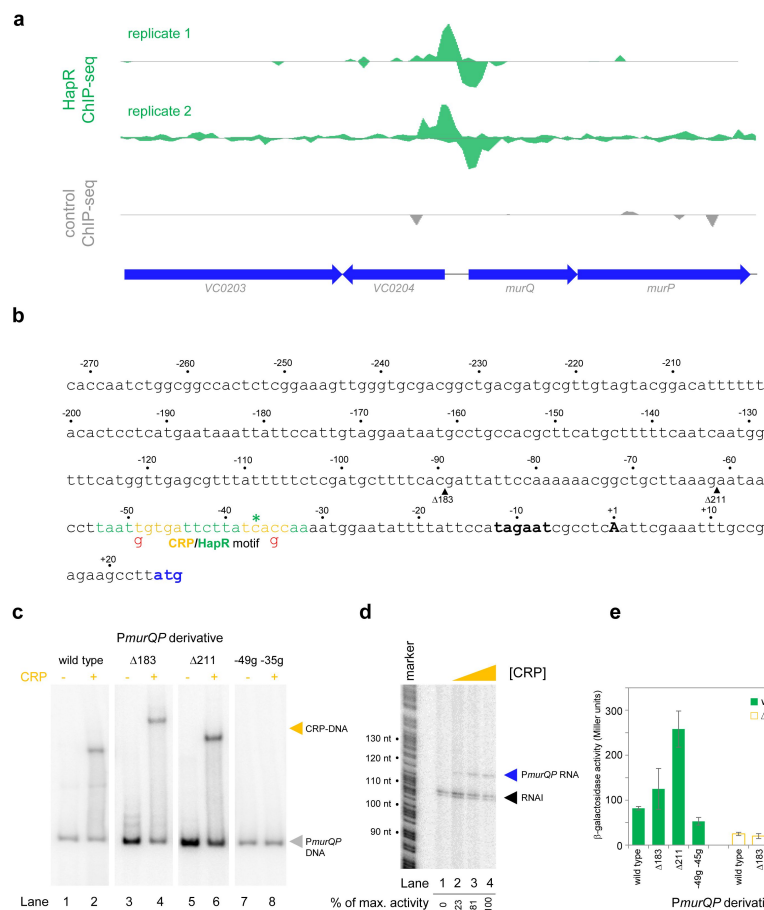


Figure 3:

Transcription from the *murQP* promoter requires CRP *in vivo* and *in vitro*.

a. HapR binding to the *murQP* regulatory region. Genes are shown as block arrows. ChIP-seq coverage plots are shown for individual experimental replicates. Signals above or below the horizontal line correspond to reads mapping to the top or bottom strand respectively.

b. DNA sequence of the intergenic region upstream of *murQP*. For clarity, numbering is with respect to the *murQP* transcription start site (TSS, +1). The TSS and promoter -10 element are in bold. The *murQ* start codon is in blue. The HapR binding site, predicted by MEME analysis of our ChIP-seq data for HapR, is in green. A potential CRP site is embedded within the HapR binding sequence (orange). Sequences in red indicate point mutations used in this work. Triangles show sites of truncation.

c. Binding of CRP to the *murQP* regulatory region and derivatives.

Electrophoretic mobility shift assays showing migration of the *murQP* regulatory region, or indicated derivatives, with or without 0.1 μ M CRP. The DNA fragment used is shown above each pair of lanes and correspond to the truncations or point mutations indicated in panel b.

d. The *murQP* promoter is activated by CRP *in vitro*. The gel image shows the result of an *in vitro* transcription assay. The DNA template was plasmid pSR carrying the *murQP* regulatory region. Experiments were done with 0.4 μ M RNA polymerase with or without 0.125, 0.25, or 0.5 μ M CRP. The RNAI transcript is plasmid-derived and acts as an internal control.

e. The *murQP* promoter is activated by CRP *in vivo*. The bar chart shows results of β -galactosidase activity assays. Cell lysates were obtained from wild type *V. cholerae* E7946 (solid green) or the Δ crp derivative, transformed with pRW50T derivatives containing the indicated promoter derivatives fused to lacZ. Standard deviation is shown for three independent biological replicates. Cells were grown in LB-Lennox medium at 37°C to an OD₆₅₀ of ~1.1.

Transcription from the *murQP* promoter requires CRP in vivo and in vitro

The *murQP* operon (VC0206-VC0207) encodes functions important for recycling of peptidoglycan⁽³³⁾. Briefly, cell wall derived *N*-acetylmuramic acid (MurNAc) is transported across the inner membrane, and simultaneously phosphorylated, by the phosphotransferase system dependent permease MurP. Resulting MurNAc-6P is hydrolysed by MurQ to generate *N*-acetylglucosamine 6-phosphate (GlcNAc-6P). Intriguingly, GlcNAc-6P can also be derived

from chitin break down and this coincides with expression of HapR. Hence, we focused on understanding the role of HapR bound upstream of *murQP*. The HapR ChIP-seq binding signal at the *murQP* locus is shown in Figure 3a and the associated regulatory region is shown in Figure 3b. The centre of the ChIP-seq peak for HapR is marked by an asterisk and the predicted binding site is highlighted green. We reasoned that our inability to detect transcription from *PmurQP* *in vitro* was likely because an undefined transcriptional activator is absent (Figure 2b). Inspection of the DNA sequence upstream of *murQP* identified a close match to the consensus binding site for CRP (5'-TGTGA-N₆-TCACA-3'). Furthermore, this sequence was located 41.5 bp upstream, of the *murQP* TSS (Figure 3b). This is a common scenario for CRP dependent transcription activation⁽³⁴⁾. To measure binding of CRP to the *murQP* regulatory region we used electrophoretic mobility shift assays (EMSAs). Consistent with our prediction, CRP bound to the *murQP* regulatory DNA (Figure 3c, lanes 1 and 2). To confirm that we had correctly identified the binding site for CRP we made a series of *PmurQP* derivatives. The Δ183 and Δ211 DNA fragments have large upstream deletions (sites of truncation are shown by inverted triangles in Figure 3b, which mark the 5' end of the remaining promoter DNA) but still bind CRP (Figure 3c, lanes 3-6). Conversely, point mutations -35g and -49g, within the CRP site, prevent binding (Figure 3c, lanes 7-8). To determine the impact of CRP on *PmurQP* activity we first used *in vitro* transcription assays (Figure 3d). Addition of CRP to reactions resulted in production of an RNA from *PmurQP*. We observed similar CRP dependence *in vivo* using β-galactosidase assays (Figure 3e, compare wild type promoter activity with and without CRP). Furthermore, in wild type cells, the -35g and -49g mutations reduced promoter activity whilst the Δ183 and Δ211 truncations did not (Figure 3e). We note that the Δ211 derivative is much more active than the starting promoter DNA sequence, but transcription remains totally dependent on CRP. Most likely, the truncation removes a repressive DNA element upstream of the core promoter.

HapR and CRP bind a shared DNA site at the *murQP* promoter

At *PmurQP*, the DNA site for CRP is completely embedded within the predicted HapR binding sequence (Figure 3b). To better understand this unusual configuration, we used DNaseI footprinting. The results are shown in Figure 4a. Lane 1 shows the pattern of DNaseI digestion in the absence of bound protein. In the presence of CRP (lanes 2-4) a footprint was observed between positions -29 and -59 bp relative to the *murQP* TSS. As is usual for CRP, and a consequence of DNA bending, the footprint comprised protection from, and hypersensitivity to, DNase I attack. Three distinct sites of DNaseI hypersensitivity are marked by orange arrows alongside lane 4 in Figure 4a. The pattern of DNase I digestion in the presence of HapR is shown in lanes 5-8. The footprint due to HapR binding exactly overlaps the region bound by CRP and results in complete protection of the DNA from digestion between positions -29 and -58 (green bar adjacent to lane 8). We also observed changes in the relative intensity of bands upstream of the HapR site between promoter positions -60 and -80. We speculate that this may result from changes in DNA conformation. Importantly, there was one further subtle difference between HapR and CRP induced banding patterns. Namely, in the presence of HapR, a band was observed at position -58 (see green triangle adjacent to lane 8). With CRP, a band was instead observed at position -59 (compare lanes 2-4 with 5-8). In a final set of assays, we examined addition of CRP and HapR in unison. We reasoned that 3 outcomes were possible. First, one of the two protein factors could outcompete the other. This should result in a DNase I digestion pattern identical to either the individual CRP or HapR footprint. Second, some DNA fragments in the reaction could be bound by CRP and others by HapR. In this case, a mixed DNase I digestion pattern, containing all features of the individual footprints due to CRP and HapR, should occur. Third, CRP and HapR could bind simultaneously. This might generate a DNase I digestion pattern with similarities to the CRP and HapR footprints. However, accessibility of the

nucleic acid to DNase I would likely be altered in some way, with unpredictable outcomes. The result of the experiment was analysed in lanes 9-12. The binding pattern matched only some aspects of the individual footprints for CRP and HapR. Hence, we observed 2 of the 3 DNase I hypersensitivity sites due to CRP binding. Changes in the banding pattern upstream of the binding sequence, due to HapR, were also detected. We did not observe the band at position -58 detected with HapR alone. Rather, we observed a band at position -59. An additional band at position -26 (black triangle adjacent to lane 12) was unique to these reactions. We conclude that HapR and CRP recognise the same section of the *murQP* regulatory region and may bind in unison.

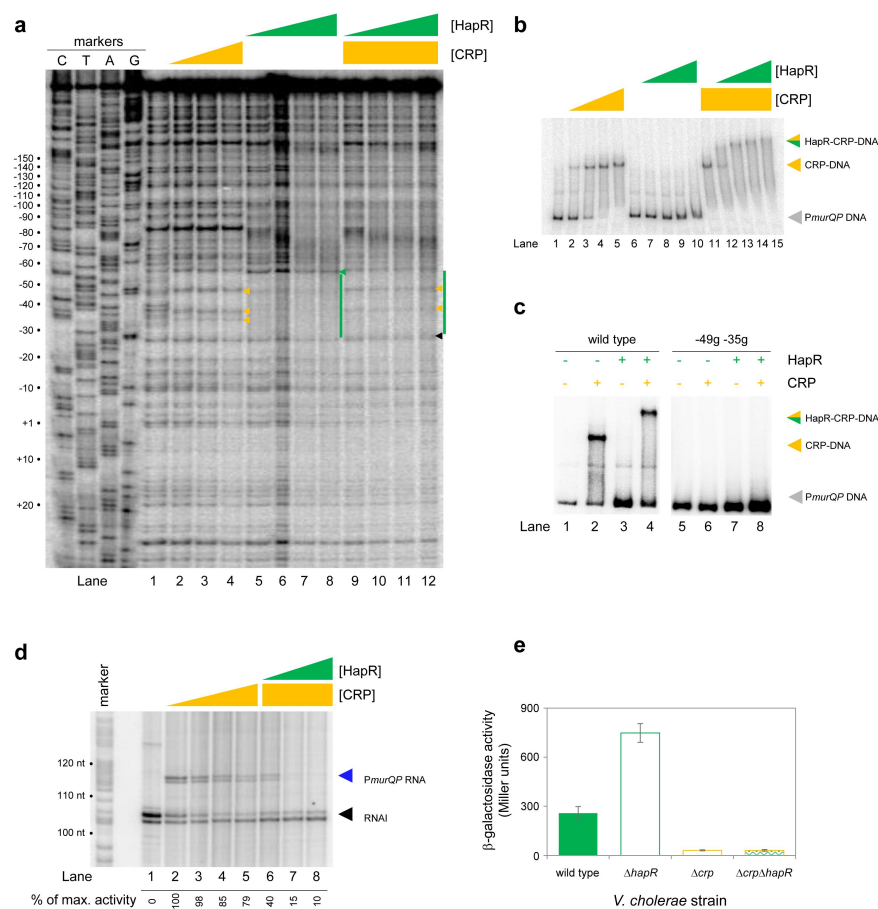


Figure 4:

HapR and CRP co-operatively bind the same section of *murQP* regulatory DNA.

a. Binding locations of HapR and CRP upstream of *murQP*. The gel shows the result of DNase I footprinting experiment. The gel is calibrated with Sanger sequencing reactions. The pattern of DNase I cleavage in the absence of any proteins is in lane 1. Protection of DNA from DNase I cleavage in the presence of 0.11, 0.23 or 0.45 μ M CRP is shown in lanes 2-4. Sites of DNase I hypersensitivity due to CRP binding are indicated by orange triangles. Protection from DNase I cleavage in the presence of 0.5, 1.0, 2.0 or 3.0 μ M HapR is shown in lanes 5-8. Protection from DNase I cleavage, dependent on HapR, is shown by a green bar. A DNase I hypersensitive band, unique to reactions with HapR, is shown by a green triangle. In the presence of 0.45 μ M CRP, increasing

concentrations of HapR result in a different DNase I cleavage pattern, including the appearance of a different site of hypersensitivity (black triangle).

b. Binding of HapR and CRP upstream of *murQP* is co-operative. Electrophoretic mobility shift assays showing migration of the *murQP* regulatory region with different combinations of CRP (0.025, 0.05, 0.1 or 0.2 μ M) and HapR (0.5, 1.0, 2.0, 3.0 or 4.0 μ M). For incubations with both factors, the same range of HapR concentrations was used with 0.2 μ M CRP.

c. Co-operative binding of CRP requires the shared HapR and CRP binding site. Results of an electrophoretic mobility shift assay, using the wild type *murQP* regulatory region or a derivative with two point mutations in the shared recognition sequence, for HapR (4.0 μ M) and CRP (0.1 μ M). Positions of mutations are shown in Figure 3b.

d. HapR blocks CRP mediated activation of the *murQP* promoter *in vitro*. The gel image shows the result of an *in vitro* transcription assay. The DNA template was plasmid pSR carrying the *murQP* regulatory region. Experiments were done with 0.4 μ M RNA polymerase, with or without 0.05, 0.1, 0.2 or 0.5 μ M CRP 0.5, 1.0, 2.0 or 3.0 μ M HapR, as indicated. The RNAI transcript is plasmid-derived and acts as an internal control.

e. HapR represses CRP mediated activation of the *murQP* promoter *in vivo*. β -galactosidase activity was measured in cell lysates taken from *Vibrio cholerae* E7946 (solid green bars), $\Delta hapR$ derivative (open green bars), Δcrp variant (open orange bars), or cells lacking both factors (orange outline with green patterned fill). Standard deviation is shown for three independent biological replicates. Cells were grown in LB-Lennox medium to an OD_{650} of ~ 1.0 at 37°C .

HapR and CRP bind the *murQP* promoter co-operatively

Fragments of the *murQP* regulatory DNA, simultaneously bound by CRP and HapR, are expected to have distinct migratory properties during electrophoresis. Thus, we compared binding of CRP and/or HapR using EMSAs. The results are shown in [Figure 4b](#). As expected, addition of CRP to reactions caused a distinct shift in electrophoretic mobility (lanes 1-5). Comparatively, at the concentration used, HapR bound the DNA fragment poorly; we observed only smearing of the free DNA at the highest HapR concentration tested (lanes 6-10). The binding pattern due to HapR was dramatically different if DNA was pre-bound with CRP (lanes 11-15). In this scenario, even low concentrations of added HapR were sufficient to generate a super-shifted nucleoprotein complex (lanes 11-15). These data are consistent with HapR having a higher affinity for CRP-*PmurQP* than *PmurQP* alone. Hence, HapR and CRP bind the *murQP* regulatory region co-operatively. A mundane explanation is that increased molecular crowding, upon CRP addition, increases the effective concentration of HapR. To exclude this possibility, we did two further sets of EMSA experiments. In the first set of assays, CRP was added at a lower concentration. Thus, some DNA remained unbound ([Figure 4c](#), lanes 1 and 2). Hence, when added to such reactions, HapR could bind either the free DNA or the CRP-DNA complex. Consistent with HapR preferentially binding the latter, all of the CRP-DNA complex was super shifted upon HapR addition. Conversely, the free DNA remained unbound (compare lanes 2 and 4). In equivalent experiments, with point mutations -49g and -35g in the CRP site, neither CRP or HapR were able to bind the DNA (lanes 5-8). In a second set of tests, we used the *hapR* regulatory DNA that binds HapR but not CRP. If CRP addition increased the effective concentration of HapR, this should result in much tighter HapR binding to the *hapR* promoter. However, this was not the case ([Figure 4-figure supplement 1](#)). Taken together, our data are consistent with CRP and HapR co-operatively binding the same DNA locus at the *murQP* promoter region.

HapR represses CRP dependent transcription from the *murQP* promoter *in vivo* and *in vitro*

Recall that, in the absence of CRP, *PmurQP* is inactive *in vitro* ([Figures 2b](#) and [3d](#)). Furthermore, the promoter is subject to repression by HapR *in vivo* ([Figure 2a](#)). An explanation consistent with both observations is that HapR directly counteracts CRP mediated activation. To test this, we used *in vitro* transcription assays ([Figure 4d](#)). As expected, addition of CRP activated *murQP* transcription (lanes 1-4) and this was blocked by addition of HapR (lanes 5-8). We also repeated our prior *lacZ* fusion experiments, using the $\Delta 211$ *PmurQP* derivative, and *V. cholerae* E7946 lacking *crp* and/or *hapR*. The result is shown in [Figure 4e](#). Deletion of *hapR* caused increased transcription from *PmurQP* only when CRP was present. Hence, HapR also represses CRP dependent *murQP* transcription *in vivo*.

Binding sites for CRP and HapR overlap in a specific configuration genome-wide

Both CRP and HapR bind the same DNA region upstream of *murPQ*. This suggests similar nucleic acid sequences are recognised by each factor. Figure 5a shows an alignment of DNA logos, derived from CRP⁽⁹⁾ (top) and HapR (bottom) ChIP-seq targets. The two motifs have features in common that align best when the logo centres are offset by 1 base pair. This is consistent with the arrangement of binding sites upstream of *murPQ* (Figure 3b). To understand the importance of this configuration we first took a bioinformatic approach. The DNA sequences logos shown in Figure 5a were used to create position weight matrices (PWMs) describing either the CRP or HapR binding site. We then searched the *V. cholerae* genome, using each PWM, and calculated the distance between identified CRP and HapR sites. The data for all sites within 100 bp of each other is shown in Figure 5b (top panel). In all cases, the CRP and HapR targets were offset by 1 bp. We then repeated the analysis after randomising the *V. cholerae* genome sequence (bottom panel). The number of overlapping targets was reduced 7-fold. An equivalent analysis of the *V. harveyi* genome produced similar results (Figure 5-figure supplement1). Hence, sites for CRP and HapR have a propensity to coincide in a specific configuration. That such sites are found more frequently in native genome sequences, compared to those first randomised, suggests selection during genome evolution. We next sought to understand how this arrangement might permit simultaneous and co-operative binding of CRP and HapR.

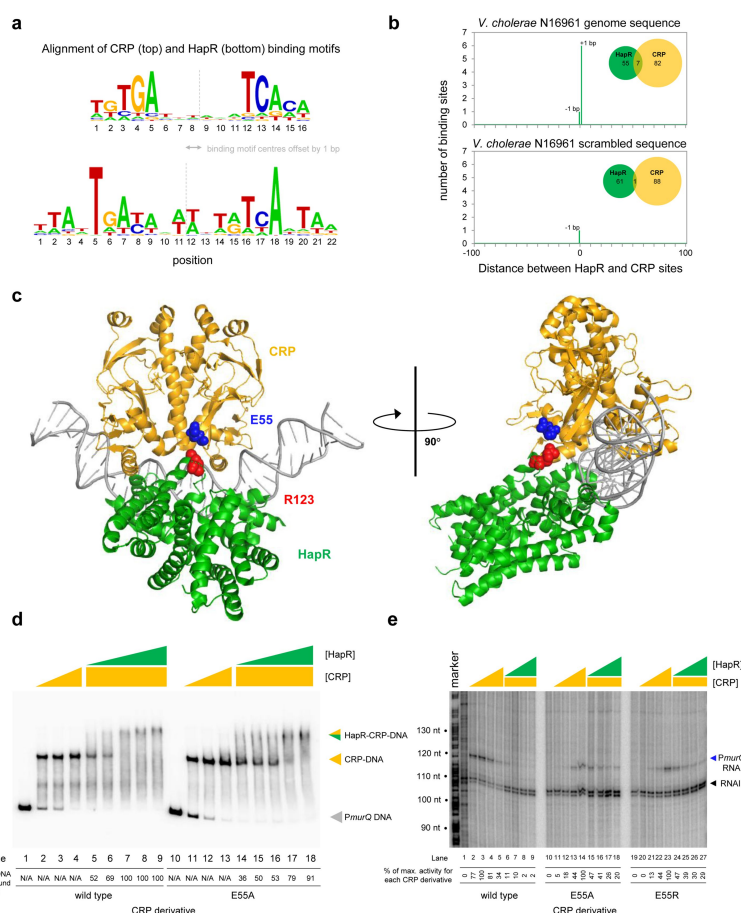


Figure 5:

HapR contacts Activation Region 3 of CRP.

a. Binding sites for CRP and HapR are optimally aligned when offset by one base pair.

The panel shows DNA sequences logos generated by aligning binding sites identified by ChIP-seq analysis for CRP (top) and HapR (bottom). The centre of each motif is indicated by a dashed line.

b. Global overlap of CRP and HapR binding sites. A position weight matrix (PWM), corresponding to each DNA sequence logo shown in panel a, was created. The PWMs were used to search the *V. cholerae* genome sequence using FIMO. Distances between the identified CRP and HapR sites were calculated. Proximal sites were always overlapping and offset by one base pair (top panel). Overlap was greatly reduced when the analysis was applied to a randomised version of the same genome sequence (bottom panel).

c. Model of the DNA-CRP-HapR complex. The model was generated using PDB submissions 6pb6 (*E. coli* CRP in complex with a class II CRP dependent promoter) and 1jt0 (*S. aureus* QacR bound to its DNA target).

Note that QacR is closely related to *V. cholerae* HapR. The structures were aligned so that the CRP and HapR binding centres were offset by one base pair. Residue E55 of CRP (blue) is within Activating Region 3 of CRP that can interact with the RNA polymerase sigma subunit at class II promoters. HapR residue R123 (red) participates in HapR dimerisation and is proximal to E55 of CRP.

d. Side chain E55 of CRP is required for stability of the DNA-CRP-HapR complex. Electrophoretic mobility shift assays showing migration of the *murQP* regulatory region with different combinations of CRP or CRP^{E55A} (0.15, 0.3 or 0.6 μ M) and HapR (0.083, 0.125, 0.166 0.208 or 0.25 μ M).

e. HapR cannot repress transcription activated by CRP^{E55A}. Result of an *in vitro* transcription assay. The DNA template was plasmid pSR carrying the *murQP* regulatory region. Experiments were done with 0.4 μ M RNA polymerase, with or without 0.05, 0.1, 0.2 or 0.5 μ M CRP or CRP^{E55A} and 0.5, 1.0, 2.0 or 3.0 μ M HapR, in the presence of 0.2 μ M CRP, as indicated. The RNAI transcript is plasmid-derived and acts as an internal control.

A structural model of the DNA-CRP-HapR ternary complex

To understand organisation of the DNA-CRP-HapR ternary complex we used structural modelling. The *V. cholerae* CRP protein is 96 % identical to the equivalent factor in *Escherichia coli*. Similarly, the *Staphylococcus aureus* factor QacR is 50 % similar to *V. cholerae* HapR. Previously, structural biology tools were used to investigate *E. coli* CRP, and *S. aureus* QacR, bound with their cognate DNA targets. We used this information to build a model for the DNA-CRP-HapR ternary complex. Importantly, we ensured that the CRP and HapR binding centres were offset by 1 bp. When aligned in this way, CRP and HapR recognise the same section of DNA via different surfaces of the double helix. We examined the model in the context of our DNase I footprinting data. Recall that CRP binding upstream of *murPQ* induces three sites of DNase I hypersensitivity (Figure 4a). These correspond to positions -47, -38 and -34 with respect to the *murQP* TSS. Figure 5-figure supplement 2 shows these positions highlighted in the context of our model. In the presence of CRP alone, all sites are surface exposed but position -34 is partially occluded by CRP (Figure 5-figure supplement 2a). This likely explains why positions -47 and -38 are more readily cleaved by DNase I (Figure 4a). With both CRP and HapR, position -34 was completely protected from DNase I attack (Figure 4a). Consistent with the footprinting data, our model indicates that position -34 is almost completely hidden upon binding of HapR (Figure 5-figure supplement 2b). Conversely, access to positions -47 and -38 is not altered (Compare Figure 5-figure supplement 2a and 2b).

Co-operative binding with HapR requires CRP residue E55

Co-operative DNA binding by transcription factors can result from their direct interaction^{(35)–(37)}. In our model, a negatively charged surface of CRP (including residue E55) is in close proximity to positively charged HapR residue R123 (Figure 5c). In initial experiments, we mutated both protein surfaces to remove the charged side chain, or replace the residue with an oppositely charged amino acid. We then investigated consequences for HapR and CRP binding individually at *PmurQP* using EMSAs (Figure 5-figure supplement 3). Whilst the CRP derivatives were able to bind the *murQP* regulatory region normally, HapR variants were completely defective. This is likely because R123 sits at the HapR dimerisation interface. Hence, we focused on understanding the contribution of CRP sidechain E55 to co-operative DNA binding by HapR and CRP using EMSAs. The results are shown in Figure 5d. Both wild type CRP, and CRP^{E55A}, were able to bind the *murQP* regulatory region similarly (lanes 1-4 and 10-13). As expected, HapR bound tightly to the wild type CRP:DNA complex

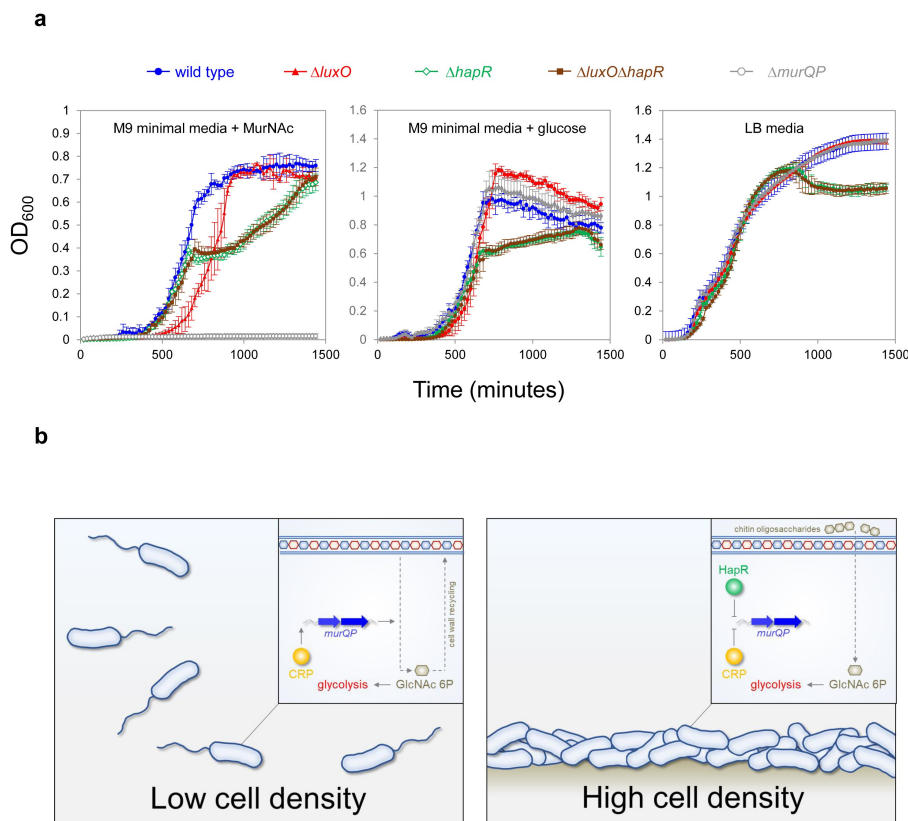
(lanes 5-9). Conversely, HapR had a lower affinity for DNA in complex with CRP^{E55A} (lanes 14-18). This suggests that the E55A mutation in CRP destabilises the interaction with HapR.

Repression of *PmurQP* by HapR requires CRP residue E55

Residue E55 locates to a negatively charged surface of CRP called Activating Region 3 (AR3). This determinant aids recruitment of RNA polymerase when CRP binds close to the promoter -35 element⁽³⁸⁾. Hence, AR3 is likely to be important for activation of *PmurQP* (Figure 3b). We inferred that CRP lacking E55 should activate *PmurQP* less efficiently but be less sensitive to negative effects of HapR. To test these predictions, we used *in vitro* transcription assays. The results for CRP, CRP^{E55A} and CRP^{E55R} are shown in Figure 5e. All CRP derivatives were able to activate transcription from *PmurQP*. However, consistent with an important role for AR3, the ability of the CRP^{E55A} and CRP^{E55R} to activate transcription was impaired (compare lanes 1-5, 10-14 and 19-23). Crucially, whilst HapR reduced transcription dependent on wild type CRP by 50-fold (compare lane 4 with lanes 6-9) only a 2-fold effect of HapR was observed with CRP^{E55A} (compare lane 13 with lanes 15-18). In the presence of CRP^{E55R}, HapR was even less effective (compare lane 22 with lanes 24-27).

High cell density locked *V. cholerae* are defective for growth on MurNAc

Phosphorylated LuxO activates expression of the Qrr sRNAs that inhibit *hapR* expression at low cell density (Figure 1a). Consequently, deletion of *luxO* causes constitutive expression of HapR. Thus, $\Delta luxO$ *V. cholerae* are “locked” in a high cell density state⁽²⁹⁾. Our model predicts that such strains will be defective for growth using MurNAc as the sole carbon source, as this requires expression of *murQP* that is repressed by HapR. Furthermore, any such defect should be relieved upon deletion of *hapR*. To test this, we constructed strains lacking different combinations of *luxO* and *hapR*. We also tested a *V. cholerae* derivative lacking *murP*⁽³⁹⁾. Figure 6a illustrates growth in M9 minimal media, supplemented with MurNAc or glucose, and in LB-Miller medium. As expected, cells lacking *murP* could not grow when MurNAc was the only carbon source but were not defective in other conditions (compare grey data points in each panel). Cells lacking *hapR*, alone or in combination with *luxO*, had a similar growth defect in all conditions. Strikingly, the *luxO* mutant (high cell density locked), exhibited a growth defect only when MurNAc was the sole carbon source (compare red data points).



HapR. In low *V. cholerae* population density conditions (left panel) cell division necessitates cell wall turnover. Expression of MurQP facilitates cell wall recycling and conversion of MurNac to GlcNAc 6P for glycolysis (insert). At high cell density conditions (right panel) *V. cholerae* form biofilms on chitinous surfaces. Reduced cell division, and the availability of chitin derived GlcNAc 6P, reduces the need for MurQP.

Specifically, these cells exhibited an extended lag phase in MurNac. This extended lag phase was not apparent when both *luxO* and *hapR* were deleted, consistent with the effect of *luxO* being mediated by HapR-dependent repression of *murQP*.

Co-operative interactions between HapR and CRP are commonplace

In a final set of experiments, we turned our attention to other sites shared by CRP (prior work⁽⁹⁾) and HapR (Table 1). We selected 5 such targets and examined binding of CRP and HapR using EMSAs. At 1 target, adjacent to *VCA0218*, binding was not co-operative and free DNA remained when both proteins were present (Figure 5-figure supplement 4). For 4 of the targets, we detected co-operative binding of CRP and HapR, reminiscent of our experiments with *PmurQP* DNA (compare Figure 5-figure supplement 4 and Figure 4). At these loci (adjacent to *VC0102*, *VC1851*, *VCA0663* and *VCA0691*) either HapR or CRP bound poorly to DNA in the absence of the other protein. However, when both factors were added together, all DNA shifted into a distinct low mobility complex. We conclude that co-operative binding of HapR and CRP to shared targets is common.

Figure 6:

Control of *murQP* expression by CRP and HapR at low and high cell density.

a. *V. cholerae* locked at high cell density are defective for growth using MurNac as the sole carbon source. Each panel illustrates the optical density of *V. cholerae* cultures at different timepoints after inoculation. Cells were grown at 32 °C in M9 minimal media supplemented with the indicated carbon source (0.25 % w/v) or LB-Miller medium. Cells lacking *luxO*, but not *luxO* and *hapR*, mimic the high cell density state. Error bars show standard deviation from three separate experimental replicates.

b. Model for coordination of MurNac catabolism by CRP and

Discussion

Previously, two studies have mapped DNA binding by HapR homologs in *Vibrio* species. For *V. harveyi*, van Kessel and co-workers used ChIP-seq to identify 105 LuxR binding targets⁽³⁰⁾. At 77 of these sites, LuxR repressed transcription. Using ChIP-seq and global DNase I footprinting, Zhang *et al.* found 76 LuxR bound regions in *Vibrio alginolyticus*⁽³¹⁾. Regulatory effects were evident for 37 targeted genes, with 22 cases of LuxR mediated repression. In the present study, we identified 32 HapR bound sections of the *V. cholerae* genome. Consistent with prior work, repression of target genes was the most common regulatory outcome. Furthermore, the DNA binding consensus derived here for HapR is almost identical to motifs for LuxR binding in *V. harveyi* and *V. alginolyticus*. Contrastingly, Tsou and colleagues used bioinformatic tools to predict HapR binding in *V. cholerae*⁽²⁸⁾. Two different HapR binding motifs were proposed. Both partially match the HapR target sequence proposed here. Most likely, the analysis of Tsou *et al.* was hampered by a paucity of targets from which a full consensus could be derived. We note that our list of 32 HapR targets does not include all known targets. However, on inspection, whilst insufficient to pass our stringent selection criteria, weaker signals for HapR are evident at many such locations (Figure 1-figure supplement 2 and Supplementary file 1). In particular, we note evidence for binding of HapR upstream of *hapA*, which has previously been only inferred (Figure 1-figure supplement 2b). We note that previous relied on computational predictions and *in vitro* DNA binding assays to identify potential HapR targets. That not all such targets are bound *in vivo*, in the single growth condition tested here, is to be expected.

Recognition of shared DNA targets provides a simple mechanism for integration of quorum sensing signals, relayed by HapR, and cAMP fluctuations, communicated by CRP. In the example presented here, HapR acts to prevent transcription activation by co-binding the same DNA target with CRP (Figure 4). Hence, at *PmurQP*, the function of CRP switches from that of an activator to a co-repressor with HapR (Figure 6b). This regulatory strategy is a logical consequence of *V. cholerae* forming biofilms on chitinous surfaces. At low cell density, rapidly dividing cells must continually remodel their cell wall. In these conditions, HapR is not expressed. Thus, MurQ and MurP are produced and can convert cell wall derived MurNAc to GlcNAc-6P. Conversely, in high cell density scenarios, usually involving adherence to chitin, cells divide infrequently, and remodelling of the cell wall is reduced. In addition, GlcNAc-6P can be derived readily from chitin oligosaccharides. Hence, cells locked in the high cell density state are defective for growth when supplied with MurNAc as the sole carbon source (Figure 6a). We suggest that HapR and CRP are likely to coordinate the expression of other metabolic enzymes in a similar way. Interestingly, AphA, another quorum sensing responsive regulator, also acts alongside CRP at many *V. cholerae* promoters⁽⁴⁰⁾. Indeed, AphA and CRP binding sites can overlap but this results in competition between the factors⁽⁴⁰⁾. Together with results presented here, these observations highlight close integration of quorum sensing with gene control by cAMP in *V. cholerae*.

Materials and methods

Strains, plasmids and oligonucleotides

Strains, plasmids and oligonucleotides used in this study are listed in Supplementary file 2. All *V. cholerae* strains are derivatives of E7946⁽⁴¹⁾. Chromosomal deletions were made using the pKAS32 suicide plasmid for allelic exchange⁽⁴²⁾,⁽⁴³⁾ or via splicing-by-overlap-extension

PCR and chitin-induced natural transformation⁽⁴⁴⁾. The *E. coli* strain JCB387 was used for routine cloning⁽⁴⁵⁾. Plasmids were transferred into *V. cholerae* by either conjugation or transformation as described previously^{(9), (40)}.

ChIP-seq and bioinformatics

Chromatin immunoprecipitation was done as in prior work⁽⁴⁰⁾ using strain E7946, carrying plasmid pAMCF-*luxO* or pAMNF-*hapR*, and anti-FLAG antibodies. In both cases, control experiments were done using the equivalent plasmid with no gene insert. Note that both plasmids drive low level constitutive expression of 3xFLAG transcription factor derivatives⁽⁴⁶⁾. Lysates were prepared from LB-Lennox medium cultures, incubated with shaking at 37 °C to an OD₆₅₀ of ~1.1. Following sonication, the protein-DNA complexes were immunoprecipitated with an anti-FLAG antibody (Sigma) and Protein A sepharose beads. Immunoprecipitated DNA was blunt-ended, A-tailed, and ligated to barcoded adaptors before elution and de-crosslinking. ChIP-seq libraries were then amplified by PCR and purified. Library quality was assessed using an Agilent Tapestation 4200 instrument and quantity determined by qPCR using an NEBnext library quantification kit (NEB). Libraries were sequenced as described previously⁽⁴⁶⁾ and reads are available from ArrayExpress using accession code E-MTAB-11906. Single-end reads, from two independent ChIP-seq experiments for each strain, were mapped to the reference *V. cholerae* N16961 genome (chromosome I: NC_002505.1 and chromosome II: NC_002506.1) with Bowtie 2⁽⁴⁷⁾. The read depth at each position of the genome was determined for each BAM file using multibamsummary. Each binding profile was then normalised to an average genome-wide read depth of 1 read per base. Following normalisation, the average read depth per base for each pair of replicates was calculated. The resulting files were used to generate the circular plots in Figure 1 using DNAPlotter⁽⁴⁸⁾. For peak selection, the files were viewed as graphs using the Artemis genome browser⁽⁴⁹⁾. After visually identifying an appropriate cut-off, peaks were selected using the “create features from graph” tool. Note that our cut-off was selected to identify only completely unambiguous binding peaks. Hence, weak or less reproducible binding signals, even if representing known targets, were excluded (see Discussion for further details). For HapR, the window size, minimum feature size, and cut-off value were 100, 100 and 10 respectively. For LuxO, the equivalent values were 100, 100 and 4. The mid-point of features selected in this way was set as the peak centre. In each case, 300 bp of sequence from the peak centre was selected and the combined set of such sequences for each factor were analysed using MEME to generate DNA sequence logos⁽⁵⁰⁾.

β-galactosidase assays

Promoter DNA was fused to *lacZ* in plasmid pRW50T that can be transferred from *E. coli* to *V. cholerae* by conjugation⁽⁹⁾. Assays of β-galactosidase activity were done according to the Miller method⁽⁵¹⁾. Bacterial cultures were grown at 37 °C with shaking in LB-Lennox medium, supplemented with appropriate antibiotics, to an OD₆₅₀ of ~1.1. Values shown are the mean of three independent experiments and error bars show the standard deviation.

Proteins

We purified *V. cholerae* CRP and RNA polymerase as described previously^{(9), (40)}. To generate HapR, *E. coli* T7 Express cells were transformed with plasmid pHis-tev-HapR, or derivatives, which encodes HapR with a His₆ tag and intervening site for the tobacco etch virus protease. Transformants were cultured in 40 ml LB-Lennox medium overnight, then sub-cultured in 1 L of fresh broth, with shaking at 37 °C. When sub-cultures reached mid-log phase they were supplemented with 400 mM IPTG for 3 hours. Cells were then collected by

centrifugation, resuspended in 40 ml of buffer 1 (40 ml 25 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 M NaCl) and lysed by sonication. Inclusion bodies, recovered by centrifugation, were resuspended with 40 ml of buffer 2 (25 mM Tris-HCl pH 8.5 and 4 M urea) before the remaining solid material was again recovered and then solubilised using 40 ml of buffer 3 (25 mM Tris-HCl pH 8.5 and 6 M guanidine hydrochloride). Cleared supernatant was applied to a HisTrap HP column (GE healthcare) equilibrated with buffer A (25 mM Tris-HCl pH 8.5 and 1 M NaCl). To elute His₆-HapR, a gradient of buffer B (25 mM Tris-HCl pH 8.5, 1 M NaCl and 1 M imidazole) was used. Fractions containing His₆-HapR were pooled and the protein was transferred into buffer X (50 mM HEPES, 1 M NaCl, 1 mM DTT, 5 mM EDTA and 0.1 mM Triton X-100) by dialysis. Finally, we used Vivaspin ultrafiltration columns to reduce sample volume. The concentration of His₆-HapR was then determined.

in vitro transcription assays

Experiments were done using our prior approach⁽⁴⁰⁾. Plasmid templates were isolated from *E. coli* using Qiagen Maxiprep kits. Each *in vitro* transcription assay contained 16 µg/ml DNA template in 40 mM Tris pH 7.9, 5 mM MgCl₂, 500 µM DTT, 50 mM KCl, 100 µg/ml BSA, 200 µM ATP/GTP/CTP, 10 µM UTP and 5 µCi α-P³²-UTP. Purified HapR and CRP were added at the indicated concentrations prior to the reaction start point. In experiments where CRP was used, the protein was incubated with cAMP 37 °C prior to addition. Transcription was instigated by addition of RNA polymerase holoenzyme prepared in advance by incubation of the core enzyme with a 4-fold excess of σ⁷⁰ for 15 minutes at room temperature. After 10 minutes incubation at 37 °C, reactions were stopped by the addition of an equal volume of formamide containing stop buffer. Reactions were resolved on an 8% (w/v) denaturing polyacrylamide gel, exposed on a Bio-Rad phosphor screen then visualised on a Bio-Rad Personal Molecular Imager. To quantify transcript levels, we measured the intensity of bands corresponding to RNAI and the RNA of interest using Quantity One software. After subtracting background lane intensity, we calculated the RNA of interest to RNAI ratio. The maximum ratio was set to 100 % activity with other ratios shown a percentage of this maximum. Experiments were repeated at least twice with similar results.

Electrophoretic mobility shift assays and DNase I footprinting

Promoter DNA fragments were excised from plasmid pSR and end-labelled with γ³²-ATP using T4 PNK (NEB). EMSAs and DNase I footprints were done as previously described⁽⁴⁰⁾. Full gel images are shown in Figure 2-figure supplement 1. Experiments were repeated at least twice with similar results.

Structural modelling

The model of the ternary DNA-CRP-HapR complex was generated in PyMOL by aligning PDB depositions 1jt0 (QacR-DNA complex) and 6pb6 (CRP-DNA complex). Alignments were done manually and guided by the relative two-fold centres of symmetry for each complex. Each structure was positioned so that their DNA base pairs overlapped and binding centres were offset by 1 base pair. The Mutagenesis function of PyMOL was used to replace QacR sidechain K107, equivalent to HapR R123⁽²⁰⁾, with an arginine residue. The double helix of the QacR DNA complex is hidden in the final model.

Acknowledgements

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Materials availability statement

Strains, plasmids and oligonucleotides are available on request.

Supplementary figure legends

Figure 1-figure supplement 1: Binding of LuxO and the *qrr1* and *VC1142* loci.

a. ChIP-seq coverage plots for individual experimental replicates. Signals above or below the horizontal line correspond to reads mapping to the top or bottom strand respectively.

b. Sequence of the intergenic region between *VC1142* and *VC1143*. The LuxO target site is shown in red. Start codons for the divergent genes *VC1142* and *VC1143* are in green. Transcription start sites identified by Papenfort *et al.*⁽³²⁾ are in uppercase with arrows indicating the direction of transcription. Underlined sequences are potential promoter -10 elements for the housekeeping RNA polymerase assuming a minimal requirement for the sequence 5'-TANNNT-3'.

Figure 1-figure supplement 2: Example HapR binding signals.

a. Binding peaks for HapR that fall above our cut-off for peak selection. The HapR ChIP-seq binding profiles are shown in green and genes are shown as blue arrows.

b. Binding peaks for HapR, at known targets, that fall below our cut-off for peak selection. Binding signals for HapR are shown at known target genes. These peaks for not selected by our analysis because the signal was too weak and/or insufficiently reproducible.

Figure 2-figure supplement 1: Original gel images.

Figure 4-figure supplement 1: Binding of HapR to the hapR promoter region in the presence and absence of CRP. Electrophoretic mobility shift assay showing migration of the *hapR* regulatory region with different combinations of CRP (0.0125, 0.025, 0.05 or 0.1 μ M) and HapR (0.0625, 0.125, 0.25 or 0.5 μ M). For incubations with both factors, the same range of HapR concentrations was used with 0.1 μ M CRP.

Figure 5-figure supplement 1: Global overlap of CRP and HapR binding sites in *Vibrio harveyi*. A position weight matrix (PWM), corresponding to each DNA sequence logo shown in Figure 5a, was created. The PWMs were used to search the *V. harveyi* genome sequence (strain ATCC 33843) using FIMO. Distances between the identified CRP and HapR sites were calculated. Proximal sites were always overlapping and offset by one base pair (top panel). Overlap was greatly reduced when the analysis was applied to a randomised version of the same genome sequence (bottom panel).

Figure 5-figure supplement 2: Models of the DNA-CRP and DNA-CRP-HapR complexes.

The models were generated using PDB submissions 6pb6 (*E. coli* CRP in complex with a class II CRP dependent promoter) and 1jt0 (*S. aureus* QacR bound to its DNA target). The DNA is shown in grey and positions hypersensitive to DNase I cleave, in the context of the DNA-CRP complex, are highlighted red (Figure 4a). DNA position -34 is not cleaved by DNase I in the context of the ternary DNA-CRP-HapR complex (Figure 4a). Consistent with this, position -34 is obscured by HapR binding.

Figure 5-figure supplement 3: Binding of CRP and HapR derivatives to *PmurQP*.

The figures shows results of electrophoretic mobility shift assays with CRP and derivatives (0.1, 0.2, 0.4 or 0.8 μ M) or HapR and derivatives (0.25, 0.5, 1.0, 2.0 or 4.0 μ M).

Figure 5-figure supplement 4: Co-operative DNA binding of HapR and CRP is common.

Electrophoretic mobility shift assays showing migration of the indicated regulatory regions with different combinations of CRP (1 μ M) and HapR (0.19 μ M). For *VCA0691* the concentration of HapR was 0.57 μ M.

Source data legends

Figure 2b source data 1: Gel image TIFF file

Figure 2b source data 2: Gel image TIFF file

Figure 2b source data 3: Gel image TIFF file

Figure 2b source data 4: Gel image TIFF file

Figure 2b source data 5: Gel image TIFF file

Figure 2b source data 6: Gel image TIFF file

Figure 3c source data 1: Gel image TIFF file

Figure 3c source data 2: Gel image TIFF file

Figure 3d source data: Gel image TIFF file

Figure 4a source data: Gel image TIFF file

Figure 4b source data: Gel image TIFF file

Figure 4c source data 1: Gel image TIFF file

Figure 4c source data 2: Gel image TIFF file

Figure 4d source data: Gel image TIFF file

Figure 5c source data: Gel image TIFF file

Figure 5e source data: Gel image TIFF file

Figure 4-figure supplement 1: Gel image TIFF file

Figure 5-figure supplement 3 source data 1: Gel image TIFF file

Figure 5-figure supplement 3 source data 2: Gel image TIFF file

Figure 5-figure supplement 4 source data 1: Gel image TIFF file

Figure 5-figure supplement 4 source data 2: Gel image TIFF file

Figure 5-figure supplement 4 source data 3: Gel image TIFF file

Figure 5-figure supplement 4 source data 4: Gel image TIFF file

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Reviewer #1 (Public Review):

Original review:

This manuscript by Walker et. al. explores the interplay between the global regulators HapR (the QS master high cell density (HDC) regulator) and CRP. Using ChIP-Seq, the authors find that at several sites, the HapR and CRP binding sites overlap. A detailed exploration of the murPQ promoter finds that CRP binding promotes HapR binding, which leads to repression of murPQ. The authors have a comprehensive set of experiments that paints a nice story providing a mechanistic explanation for converging global regulation. I did feel there are some weak points though, in particular the lack of integration of previously identified transcription start sites, the lack of replication (at least replication presented in the manuscript) for many figures, some oddities in the growth curve, and not reexamining their HapR/CRP cooperative binding model in vivo using ChIP-Seq.

Review of revised version:

This revised manuscript by Walker et. al. addresses some of the editorial points and conceptual discussion, but in general, most of my suggestions (as the previous reviewer #1) for additional experimentation or addition were not addressed as discussed below. Therefore, my overall review has not changed.

1. For example, in point 1, the suggested analysis was not performed because it is not trivial. My reason for making this suggestion is that the original manuscript was limited to *Vibrio cholerae*, and the impact of the manuscript would increase if the findings here were demonstrated to be more broadly applicable. I expect papers published in eLife to have such broad applicability. But no changes were made to the manuscript in this regard. The revised version is still limited to only *Vibrio cholerae*.
2. For point 2, the activity of FLAG-tag luxO could have been simply validated in a complementation assay. Yes, they demonstrated DNA binding, but that is not the only activity of LuxO.
3. For point 7, the transcriptional fusions were not explored at different times or different media, which is also something that was hinted at by other reviewers. In regard to exploring expression at different time points, this seems particularly relevant for QS regulated genes.
4. For point 13, the authors express that doing an additional CHIP-Seq is outside of the scope of this manuscript. Perhaps that is the case, but the point of the comment is to validate the in vitro binding results with an in vivo binding assay. A targeted CHIP-Seq approach specifically analyzing the promoters where cooperative binding was observed in vitro could have addressed this point.

Reviewer #2 (Public Review):

This manuscript by Walker et al describes an elegant study that synergizes our knowledge of virulence gene regulation of *Vibrio cholerae*. The work brings a new element of regulation for CRP, notably that CRP and the high density regulator HapR co-occupy the same site on the DNA but modeling predicts they occupy different faces of the DNA. The DNA binding and structural modeling work is nicely conducted and data of co-occupation are convincing. The work seeks to integrate the findings into our current state of knowledge of HapR and CRP regulated genes at the transition from the environment and infection. The strength of the paper is the nice ChIP-seq analysis and the structural modeling and the integration of their work with other studies. The weakness is that it is not clear how representative these data are of multiple hapR/CRP binding sites or how the work integrates as a whole with the entire transcriptome that would include genes discovered by others. Overall this is a solid work that provides an understanding of integrated gene regulation in response to multiple environmental cues.

Author Response:

The following is the authors' response to the current reviews.

Reviewer #1 (Public Review):

This revised manuscript by Walker et. al. addresses some of the editorial points and conceptual discussion, but in general, most of my suggestions (as the previous reviewer #1) for additional experimentation or addition were not addressed as discussed below. Therefore, my overall review has not changed.

In our previous response, we included i) extra experimental data illustrating the reproducibility of our results and ii) added transcription start site data at the request of this reviewer. We included the information because we agreed with the reviewer that these were

important points to address. For the points raised again below, we explained why the additional analysis was unlikely to add much in terms of insight or rigour. We have elaborated further below.

1. For example, in point 1, the suggested analysis was not performed because it is not trivial. My reason for making this suggestion is that the original manuscript was limited to Vibrio cholerae, and the impact of the manuscript would increase if the findings here were demonstrated to be more broadly applicable. I expect papers published in eLife to have such broad applicability. But no changes were made to the manuscript in this regard. The revised version is still limited to only Vibrio cholerae.

Our paper is focused on the unexpected co-operative interactions between HapR and CRP. Such co-binding of two transcription factors to the same DNA site is unexpected. Consequently, it is this mode of DNA binding that is likely to be of broad interest. With this in mind, we did provide experimental, and bioinformatic, analyses for other regulatory regions and other vibrio species (Figures S3 and S6). This, in our view, is where the “broad applicability” for papers published in eLife comes from.

The analysis the reviewer suggests is not related to the main message of our paper. Instead, the reviewer is asking how many HapR binding sites seen here by ChIP-seq are also seen in other vibrio species by ChIP-seq. This is only likely to be of interest to readers with an extremely specific interest in both vibrio species and HapR. The reviewer states above that we did not make the change “because it is not trivial”. This is an oversimplification of the rationale we presented in our response. The analysis is indeed not straightforward. However, much more importantly, the outcome is unlikely to be of interest to many readers, and has no bearing on the rigour of work. With this in mind, we do not think our position is unreasonable. We also stress that, should a reader with this very specific interest want to explore further, all of our data are freely available for them to do so.

1. For point 2, the activity of FLAG-tag luxO could have been simply validated in a complementation assay. Yes, they demonstrated DNA binding, but that is not the only activity of LuxO.

DNA binding by LuxO is the only activity of the protein with which we are concerned in our paper. Furthermore, LuxO is very much a side issue; we found binding to only the known targets and potentially, at very low levels, one additional target. No further LuxO experiments were done for this reason. Indeed, even if these data were removed completely, our conclusions would not change or be supported any less vigorously. We are happy to remove the LuxO data if the reviewer would prefer but this would seem like overkill.

1. For point 7, the transcriptional fusions were not explored at different times or different media, which is also something that was hinted at by other reviewers. In regard to exploring expression at different time points, this seems particularly relevant for QS regulated genes.

In their previous review, the reviewer did not request that such experiments were done. Similarly, no other reviewer requested these experiments. Instead, this reviewer i) commented that lacZ fusions were not as sensitive as luciferase fusions ii) asked if we had done any time point experiments. We agreed with the first point, whilst also noting that lacZ is not unusual to use as a reporter. For the second point, we responded that we had not done such experiments (which by the reviewer’s own logic would have been complicated using lacZ as a reporter). This seems like a perfectly reasonable way to respond.

We should stress that these comments all refer to Figure 2a, which was our initial screening of 23 promoter::lacZ fusions, supported by separate in vitro transcription assays. Only one of these fusions was followed up as the main story in the paper. Given that the other 22 fusions

were not investigated further, and do not form part of the main story, there would seem little value in now going back to assay them at different time points.

1. For point 13, the authors express that doing an additional CHIP-Seq is outside of the scope of this manuscript. Perhaps that is the case, but the point of the comment is to validate the in vitro binding results with an in vivo binding assay. A targeted CHIP-Seq approach specifically analyzing the promoters where cooperative binding was observed in vitro could have addressed this point.

We did appreciate the original comment, and responded as such, but we do think additional ChIP-seq assays are outside the scope of this paper.

Reviewer #2 (Public Review):

This manuscript by Walker et al describes an elegant study that synergizes our knowledge of virulence gene regulation of Vibrio cholerae. The work brings a new element of regulation for CRP, notably that CRP and the high density regulator HapR co-occupy the same site on the DNA but modeling predicts they occupy different faces of the DNA. The DNA binding and structural modeling work is nicely conducted and data of co-occupation are convincing. The work seeks to integrate the findings into our current state of knowledge of HapR and CRP regulated genes at the transition from the environment and infection. The strength of the paper is the nice ChIP-seq analysis and the structural modeling and the integration of their work with other studies.

We thank the reviewer for the positive comments.

The weakness is that it is not clear how representative these data are of multiple hapR/CRP binding sites

This comment does not consider all data in our paper. We did test our model experimentally at multiple HapR and CRP binding sites. These data are shown in Figure S6 and confirm the co-operative interaction between HapR and CRP at 4 of a further 5 shared binding sites tested. We also used bioinformatics to show the same juxtaposition of CRP and HapR sites in other vibrio species (Figure S3). Hence, the model seems representative of most sites shared by HapR and CRP.

or how the work integrates as a whole with the entire transcriptome that would include genes discovered by others.

At the request of the reviewers, our revision integrated our ChIP-seq data with dRNA-seq data. No other suggestions to ingrate transcriptome data were made by the reviewers.

Overall this is a solid work that provides an understanding of integrated gene regulation in response to multiple environmental cues.

We thank the reviewer for the positive comment.

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

This manuscript by Walker et. al. explores the interplay between the global regulators HapR (the QS master high cell density (HDC) regulator) and CRP. Using ChIP-Seq, the authors find that at several sites, the HapR and CRP binding sites overlap. A detailed exploration of the murPQ promoter finds that CRP binding promotes HapR binding, which leads to repression of murPQ. The authors have a comprehensive set of experiments that paints a nice story providing a mechanistic explanation for converging global regulation.

We thank the reviewer for their positive evaluation.

I did feel there are some weak points though, in particular the lack of integration of previously identified transcription start sites

For completeness, we have now added the position and orientation or the nearest TSSs to each HapR or LuxO binding peak in Table 1 (based on Papenfort *et al.*).

the lack of replication (at least replication presented in the manuscript) for many figures,

We assume that the reviewer is referring to gel images rather than any other type of assay output (where error bars, derived from replicates, are shown). As is standard, we show representative gel images. All associated DNA binding and *in vitro* transcription experiments have been done multiple times. Indeed, comparison between figures reveals several instances of such replication (e.g. Figures 4b & 5d, Figures 4d & 5e). We have added details of repeats done to the methods section.

some oddities in the growth curve

We do not know why cells lacking *hapR* have a growth curve that appears biphasic. We can only assume that this is due to some regulatory effect of HapR, distinct from the *murQP* locus. Despite the unusual shape of the growth curve, the data are consistent with our conclusions.

and not reexamining their HapR/CRP cooperative binding model in vivo using ChIP-Seq.

We agree that these would be interesting experiments and, in the future, we may well do such work. Even without these data, our current model is well supported by the data presented (and the reviewer seems to agree with this above).

Reviewer #2 (Public Review):

This manuscript by Walker et al describes an elegant study that synergizes our knowledge of virulence gene regulation of Vibrio cholerae. The work brings a new element of regulation for CRP, notably that CRP and the high density regulator HapR co-occupy the same site on the DNA but modeling predicts they occupy different faces of the DNA. The DNA binding and structural modeling work is nicely conducted and data of co-occupation are convincing. The work could benefit from doing a better job in the manuscript preparation to integrate the findings into our current state of knowledge of HapR and CRP regulated genes and to elevate the impact of the work to address how bacteria are responding to the nutritional environment. Importantly, the focus of the work is heavily based on the impact of use of GlcNAc as a carbon source when bacteria bind to chitin in the environment, but absent the impact during infection when CRP and HapR have known roles. Further, the impact on biological events controlled by HapR integration with the utilization of carbon sources (including biofilm formation) is not explored.

We thank the reviewer for their overall positive evaluation.

The rigor and reproducibility of the work needs to be better conveyed.

Reviewer 1 made a similar comment (see above) and we have modified the manuscript accordingly.

Specific comments to address:

1. Abstract. A comment on the impact of this work should be included in the last sentence. Specifically, how the integration of CRP with QS for gene expression under specific environments impacts the lifestyle of Vc is needed. The discussion includes comments regarding the impact of CRP regulation as a sensor of carbon source and nutrition and these could be quickly summarized as part of the abstract.

We have added an extra sentence. However, we have used cautious language as we do not show impacts on lifestyle (beyond MurNac utilisation) directly. These can only be inferred.

1. Line 74. This paper examines the overlap of HapR with CRP, but ignores entirely AphA. HapR is repressed by Qrrs (downstream of LuxO-P) while AphA is activated by Qrrs. With LuxO activating AphA, it has a significant sized "regulon" of genes turned on at low density. It seems reasonable that there is a possibility of overlap also between CRP and AphA. While doing an AphA CHIP-seq is likely outside the scope of this work, some bioinformatic or simply a visual analysis of the promoters known AphA regulated genes would be interest to comment on with speculation in the discussion and/or supplement.

In short, everything that the reviewer suggests here has already been done and was covered in our original submission (see text towards the end of the Discussion). Also, we would like to point the referee to our earlier publication (Haycocks *et al.* 2019. The quorum sensing transcription factor AphA directly regulates natural competence in *Vibrio cholerae*. *PLoS Genet.* **15**:e1008362).

1. Line 100. Accordingly with the above statement, the focus here on HapR indicates that the focus is on gene expression via LuxO and HapR, at high density. Thus the sentence should read "we sought to map the binding of LuxO and HapR of V. cholerae genome at high density".

Note that expression of LuxO and HapR is ectopic in these experiments (i.e. uncoupled from culture density).

1. Line 109. The identification of minor LuxO binding site in the intergenic region between VC1142 and VC1143 raises whether there may be a previously unrecognized sRNA here. As another panel in figure S1, can you provide a map of the intergenic region showing the start codons and putative -10 to -35 sites. Is there room here for an sRNA? Is there one known from the many sRNA predictions / identifications previously done? Some additional analysis would be helpful.

We have added an extra panel to Figure S1 showing the position of TSSs relative to the location of LuxO binding. We have altered the main text to accommodate this addition..

1. Line 117. This sentence states that the CHIP seq analysis in this study includes previously identified HapR regulated genes, but does not reveal that many known HapR regulated genes are absent from Table 1 and thus were missed in this study. Of 24 HapR regulated investigated by Tsou et al, only 1 is found in Table 1 of this study. A few are commented in the discussion and Figure S7. It might be useful to add a Venn Diagram to Figure 1 (and list table in supplement) for results of Tsou et al, Waters et al, Lin et al, and Nielson et al and any others). A major question is whether the trend found here for genes identified by CHIP-seq in this study hold up across the entire HapR regulon. There should also be comments in the discussion on perhaps how different methods (including growth state and carbon sources of media) may have impacted the complexity of the regulon identified by the different authors and different methods.

We have added a list of known sites to the supplementary material (new Table S1). We were unsure what was meant by the comment “A major question is whether the trend found here for genes identified by CHIP-seq in this study hold up across the entire HapR regulon”. We have added the extra comment to the discussion re growth conditions, also noting that most previous studies relied on *in vitro*, rather than *in vivo*, DNA binding assays.

1. The transcription data are generally well performed. In all figures, add comments to the figure legends that the experiments are representative gels from n=# (the number of replicate experiments for the gel based assays). Statements to the rigor of the work are currently missing.

See responses above. We have added a comment on numbers of repeats to the methods section.

1. Line 357-360. The demonstration of lack of growth on MurNac is a nice for the impact of the work. However, more detailed comments are needed for M9 plus glucose for the uninformed reader to be reminded that growth in glucose is also impaired due to lack of cAMP in glucose replete conditions and thus minimal CRP is active. But why is this now dependent of hapR? A reminder also that in LB oligopeptides from tryptone are the main carbon source and thus CRP would be active.

We find this point a little confusing and, maybe, two issues (*murQP* regulation, and growth in general) are being conflated. In particular, we do not understand the comment “growth in glucose is also impaired due to lack of cAMP in glucose replete conditions and thus minimal CRP is active”.

Growth in glucose should indeed result in lower cAMP levels*, and hence less active CRP, but this does not impair growth. This is simply the cell’s strategy for using its preferred carbon source. If the reviewer were instead referring to some aspect of P_{murQP} regulation then yes, we would expect promoter activity to be lower because less active CRP would be available in the presence of glucose. The reviewer also comments “why is this now dependent of hapR?”. We assume that they are referring to some aspect of growth in minimal media with glucose. If so, the only hapR effect is the change in growth rate as cells enter mid-late log-phase (i.e. the growth curve looks somewhat biphasic). A similar effect is seen in all conditions. We do not know why this happens and can only conclude this is due to some unknown regulatory activity of HapR. Overall, the key point from these experiments is that loss of *luxO*, which results in constitutive hapR expression, lengthens lag phase only for growth with MurNac as the sole carbon source.

*Although in *V. fischeri* (PMID: 26062003) cAMP levels increase in the presence of glucose.

1. A great final experiment to demonstrate the model would have been to show co-localization of the promoter by CRP and HapR from bacteria grown in LB media but not in LB+glucose or in M9+glycerol and M9+MurNAc but not M9+glucose. This would enhance the model by linking more directly to the carbon sources (currently only indirect via growth curves)

This is unlikely to be as straightforward as suggested. The sensitivity of CRP binding to growth conditions is not uniform across different binding sites. For instance, the CRP dependence of the *E. coli melAB* promoter is only evident in minimal media (PMID: 11742992) whilst the role of CRP at the *acs* promoter is evident in tryptone broth (PMID: 14651625). Similarly, as noted above, in *Vibrio fischeri* glucose causes and increase in cAMP levels. (PMID: 26062003).

1. Discussion. Comments and model focus heavily on GlcNAc-6P but HapR has a regulator role also during late infection (high density). How does CRP co-operativity impact during the in vivo conditions?

We really can't answer this question with any certainty; we have not done any infection experiments in this work.

Does the Biphasic role of CRP play a role here (PMID: 20862321)?

Again, we cannot answer this question with any confidence as experimentation would be required. However, the suggestion is certainly plausible.

Reviewer #3 (Public Review):

*Bacteria sense and respond to multiple signals and cues to regulate gene expression. To define the complex network of signaling that ultimately controls transcription of many genes in cells requires an understanding of how multiple signaling systems can converge to effect gene expression and ensuing bacterial behaviors. The global transcription factor CRP has been studied for decades as a regulator of genes in response to glucose availability. It's direct and indirect effects on gene expression have been documented in *E. coli* and other bacteria including pathogens including *Vibrio cholerae*. Likewise, the master regulator of quorum sensing (QS), HapR, is a well-studied transcription factor that directly controls many genes in *Vibrio cholerae* and other *Vibrios* in response to autoinducer molecules that accumulate at high cell density. By contrast, low cell density gene expression is governed by another regulator AphA. It has not yet been described how HapR and CRP may together work to directly control transcription and what genes are under such direct dual control.*

We thank the reviewer for their assessment of our work.

*Using both in vivo methods with gene fusions to lacZ and in vitro transcription assays, the authors proceed to identify the smaller subset of genes whose transcription is directly repressed (7) and activated (2) by HapR. Prior work from this group identified the direct CRP binding sites in the *V. cholerae* genome as well as promoters with overlapping binding sites for AphA and CRP, thus it appears a logical extension of these prior studies is to explore here promoters for potential integration of HapR and CRP. Inclusion of this rationale was not included in the introduction of CRP protein to the in vitro experiments.*

We understand the reviewer's comment. However, the rationale for adding CRP was not that we had previously seen interplay between AphA and CRP (although this is a relevant discussion point, which we did make). Rather, we had noticed that there was an almost perfect CRP site perfectly positioned to activate PmurQP. Hence, CRP was added.

Seven genes are found to be repressed by HapR in vivo, the promoter regions of only six are repressed in vitro with purified HapR protein alone. The authors propose and then present evidence that the seventh promoter, which controls murPQ, requires CRP to be repressed by HapR both using in vivo and vitro methods. This is a critical insight that drives the rest of the manuscripts focus. The DNase protection assay conducted supports the emerging model that both CRP and HapR bind at the same region of the murPQ promoter, but interpret is difficult due to the poor quality of the blot.

There are areas of apparent protection at positions +1 to +15 that are not discussed, and the areas highlighted are difficult to observe with the blot provided.

We disagree on this point. The region between +1 and +15 is inherently resistant to attack by DNaseI and there are only ever very weak bands in this region (lane 1). Other than seeing small fluctuations in overall lane intensity (e.g. lanes 7-12 have a slightly lower signal throughout) the +1 to +15 banding pattern does not change. Conversely, there are dramatic changes in the banding pattern between around -30 and -60 (again, compare lane 1 to all other lanes). That CRP and HapR bind the same region is extremely clear. Also note that this is backed up by mutagenesis of the shared binding site (Figure 4c).

The model proposed at the end of the manuscript proposes physiological changes in cells that occur at transitions from the low to high cell density. Experiments in the paper that could strengthen this argument are incomplete. For example, in Fig. 4e it is unclear at what cell density the experiment is conducted.

Such details have been added to the figure legends and methods section.

The results with the wild type strain are intermediate relative to the other strains tested.

This is correct, and exactly what we would expect to see based on our model.

Cell density should affect the result here since HapR is produced at high density but not low density. This experiment would provide important additional insights supporting their model, by measuring activity at both cell densities and also in a luxO mutant locked at the high cell density. Conducting this experiment in conditions lacking and containing glucose would also reveal whether high glucose conditions mimicking the crp results.

We agree with this idea in principle but note that the output from our reporter gene, β -galactosidase, is stable within cells and tends to accumulate. This is likely to obscure the reduction in expression as cells transition from low to high cell density. Since we have demonstrated the regulatory effects of HapR and CRP both *in vivo* using gene knockouts, and *in vitro* with purified proteins, we think that our overall model is very well supported. Further experimental additions may provide an incremental advance but will not alter our overall story. Also note the unexpected increase in intracellular cAMP due to addition of glucose, in *Vibrio fischeri* (PMID: 26062003).

Throughout the paper it was challenging to account for the number of genes selected, the rationale for their selection, and how they were prioritized. For example, the authors acknowledged toward the end of the Results section that in their prior work, CRP and HapR binding sites were identified (line 321-22).

This is not quite what we say, and maybe the reviewer misunderstood, which is our fault. The prior work identified CRP sites whilst the current work identified HapR sites. We have made a slight alteration to the text to avoid confusion.

It is unclear whether the loci indicated in Table 1 all from this prior study. It would be useful to denote in this table the seven genes characterized in Figure 2 and to provide the locus tag for murPQ.

Again, we are unsure if we have confused the reviewer. The results in Table 1 are all HapR sites from the current work, not a prior study. However, some of these also correspond to CRP binding regions found in prior work.

The reviewer mentions “the seven genes characterised in Figure 2” but 23 targets were characterised in Figure 2a and 9 in Figure 2b. The “VC” numbers used in Figure 2 are the same as used in Table 1 so it is easy to cross reference between the two. We have added a footnote to Table 1, also referred to in the Figure 2 legend, to allow cross referencing between gene names and locus tags (including for *murQP* and *hapR*).

Of the 32 loci shown in Table 1, five were selected for further study using EMSA (line 322), but no rationale is given for studying these five and not others in the table.

This is not quite correct, we did not select 5 from the 32 targets listed in Table 1. We selected 5 targets from Table 1 that were also targets for CRP in our prior paper. This was the rationale.

Since prior work identified a consensus CRP binding motif, the authors identify the DNA sequence to which HapR binds overlaps with a sequence also predicted to bind CRP. Genome analysis identified a total of seven sites where the CRP and HapR binding sites were offset by one nucleotide as see with murPQ. Lines 327-8 describe EMSA results with several of these DNA sequences but provides no data to support this statement. Are these loci in Table 1?

This comment is a little difficult to follow, and we may have misunderstood, but we think that the reviewer is asking where the EMSA data referred to on lines 327-328 resides. We can see that the text could be confusing in this regard. We had referred to the relevant figure (Figure S6) on line 324 but did not again include this information further down in the description of the result. We have changed the text accordingly.

Using structural models, the authors predict that HapR repression requires protein-protein interactions with CRP. Electromobility shift assays (EMSA) with purified promoter DNA, CRP and HapR (Fig 5d) and in vitro transcription using purified RNAP with these factors (Figure 5e) support this hypothesis. However, the model proports that HapR “bound tightly” and that it also had a “lower affinity” when CRP protein was used that had mutations in a putative interaction interface. These claims can be bolstered if the authors calculate the dissociation constant (Kd) value of each protein to the DNA. This provides a quantitative assessment of the binding properties of the proteins.

The reviewer is correct that we do not explicitly provide a Kd. However, in both Figures 5d and 5e, we do provide very similar quantification. In 5d, our quantification is the % of the CRP-DNA complex bound by HapR (using either wild type or E55A CRP). Since the % of DNA bound is shown, and the protein concentrations are provided in the figure legend, information regarding Kd is essentially already present. In 5e, we show the % of maximal promoter activity. This is a reasonable way of quantifying the result. Furthermore, Kd is not a metric we can measure directly in this experiment that is not a DNA binding assay.

The concentrations of each protein are not indicated in panels of the in vitro analysis, but only the geometric shapes denoting increasing protein levels.

The protein concentrations are all provided in the figure legend. It is usual to indicate relative concentrations in the body of the figure using geometric shapes.

Panel 5e appears to indicate that an intermediate level of CRP was used in the presence of HapR, which presumably coincides with levels used in lane 4, but rationale is not provided.

There was no particular rationale for this, it was simply a reasonable way to do the experiment.

How well the levels of protein used in vitro compare to levels observed in vivo is not mentioned.

The protein concentrations that we use (in the nM to low μ M range) are very typical for this type of work and consistent with hundreds of prior studies of protein-DNA interactions. The general rule of thumb is that 1000 molecules of a protein per bacterial cell equates to a concentration of around 1 μ M. However, molecular crowding is likely to increase the effective concentration. Additionally, *in vitro*, where the DNA concentration is higher.

The authors are commended for seeking to connect the in vitro and vivo results obtained under lab conditions with conditions experienced by V. cholerae in niches it may occupy, such as aquatic systems. The authors briefly review the role of MurPQ in recycling of the cell wall of V. cholerae by degrading MurNac into GlcNac, although no references are provided (lines 146-50). Based on this physiology and results reported, the authors propose that murPQ gene expression by these two signal transduction pathways has relevance in the environment, where Vibrios, including V. cholerae, forms biofilms on exoskeleton composed of GlcNac.

We have added a citation to the section mentioned.

The conclusions of that work are supported by the Results presented but additional details in the text regarding the characteristics of the proteins used (Kd, concentrations) would strengthen the conclusions drawn. This work provides a roadmap for the methods and analysis required to develop the regulatory networks that converge to control gene expression in microbes. The study has the potential to inform beyond the sub-field of Vibrios, QS and CRP regulation.

As noted above, quantification essentially equivalent to Kd is already shown (% of bound substrate is indicated in figures and all protein concentrations are given in the figure legends).

Reviewer #1 (Recommendations For The Authors):

- 1. As similar experiments have been performed in other Vibrios, it would be interesting to do a more detailed analysis of the similarities and differences between the species. Perhaps a Venn diagram showing how many targets were found in all studies versus how many are species specific.*

We appreciate this suggestion but would prefer not to make this change. A cross-species analysis would be very time consuming and is not trivial. The presence and absence of each target gene, for all combinations of organisms, would first need to be determined. Then, the presence and absence of binding signals for HapR, or its equivalent, would need to be determined taking this into account. For most readers, we feel that this analysis is unlikely to add much to the overall story. Given the amount of effort involved, this seems a “non-essential” change to make.

- 1. Line 101-Are the FLAG tagged versions of LuxO and HapR completely functional? Can they complement a luxO or hapR deletion mutant?*

The activity of FLAG tagged HapR (LuxR in other Vibrio species) has been shown previously (e.g. PMIDs 33693882 and 23839217). Similarly, N-terminal HapR tags are routinely used for affinity purification of the protein without ill effect. We have not tested LuxO-3xFLAG for “full”

activity, though this fusion is clearly active for DNA binding, the only activity that we have measured here, since all known targets are pulled down.

1. Line 106-As the authors state later that there are additional smaller peaks for HapR that could be other direct targets, I think a brief mention of the methodology used to determine the cutoff for the 5 and 32 peaks for LuxO and HapR, respectively, would be informative here.

We have added a little more text to the methods section. The added text states “Note that our cut-off was selected to identify only completely unambiguous binding peaks. Hence, weak or less reproducible binding signals, even if representing known targets, were excluded (see Discussion for further details)”.

1. Line 118-Need a reference here to the prior HapR binding site.

This has been added.

1. Figs. 1e-What do the numbers on the x-axis refer to? Why not just present these data as bases? The authors also refer to distance to the nearest start codon, but this is irrelevant for 4/5 of the luxO targets as they are sRNAs. They should really refer to the distance to the transcription start site. Likewise, for HapR, distance to the nearest start codon is not as informative as distance to the nearest transcription start site. A recent paper used transcriptomics to map all the transcription start sites of V. cholerae, and these results should be integrated into the author's study rather than just using the nearest start codon (PMID: 25646441).

The numbers are kilo base pairs, this has been added to the axis label. We have also changed “start codon” to “gene start” (since “gene start” is also suitable for genes that encode untranslated RNAs).

Re comparing binding peak positions to transcription start sites (TSSs) rather than gene starts, this analysis would be useful if TSSs could be detected for all genes. However, some genes are not expressed under the conditions tested by PMID: 25646441, so no TSS is found. Consequently, for HapR or LuxO bound at such locations, we would not be able to calculate a meaningful position relative to the TSS. We stress that the point of the analysis is to determine how peaks are positioned with respect to genes (i.e. that sites cluster near gene 5' ends). Also note that nearest TSSs are now shown in the revised Table 1. In some cases, these are unlikely to be the TSS actually subject to regulation (e.g. because the regulated gene is switched off).

1. Fig. 1e-Is there directionality to the site? In other words, if a HapR binding site is located between two genes that are transcribed in opposite directions, is there a way to predict which gene is regulated? It looks like this might be the case with the list presented in Table 1, but how such determination is made and what the various symbols in Table 1 mean are not clear to me. This also has ramifications for Fig. 2a as the direction to construct the fusion is critical for the experiment.

The site is a palindrome so lacks directionality. The best prediction re regulation is likely to be positioning with respect to the nearest TSS (which is now included in Table 1). However, this would remain just a prediction and, where TSSs are in odd locations with respect to binding sites (taking into account the caveats above) predictions would be unreliable.

We are unsure which symbol the reviewer refers to in Table 1, a full explanation of any symbols used is provided in the table footnotes.

With respect to Figure 2a, if sites were between divergent genes, and met our other criteria, we tested for regulation in both directions. For example, see the divergent genes VCA0662 (classified inactive) and VCA0663 (classified repressed).

1. Fig. 2a-It is a little disappointing that the authors use LacZ fusions to measure transcription as this is not the most sensitive reporter gene. Luciferase gene fusions would have been much more sensitive. Also, did the authors examine multiple time points. The methods only describe "mid-log phase" but some of the inactive promoters could be expressed at other time points. Also, it would be simple to repeat this experiment in different media, such as minimal with glucose or another non- CRP carbon source, to expand which promoters are expressed.

The reviewer is correct regarding the sensitivity of β -galactosidase, which is very stable and so accumulates as cells grow. Even so, this reporter has been used very successfully, across thousands of studies, for decades. We did not examine multiple timepoints. We appreciate that the 23 promoter::lacZ fusions could be re-examined using varying growth conditions but this is unlikely to impact the overall conclusions, though it could generate some new leads for future work.

1. Fig. 2a legend-typos

This has been corrected.

1. Line 138-I think you mean Fig. 2a here.

This has been corrected.

1. Fig. 2b and many additional figures quantify band intensity but do not show any replication or error. Therefore, it is impossible to gauge reproducibility of these experiments.

We have added a reproducibility statement (all experiments were done multiple times with similar results) as is standard throughout the literature. Also note that there is a lot of internal replication between figures. Figure 4d and Figure 5e lanes 1-9 show essentially the same experiment (albeit with slightly different protein concentrations) and very similar results. To the same effect, Figure 5e lanes 10-18 and lanes 19-27 show the same experiment for two different mutations of the same CRP residue. Again, the results are very similar. Also see the response to your comment 15 below.

1. Fig. 4a-lanes 2-4-the footprint does not change with additional CRP. In other words, it looks the same at the lowest concentration of CRP versus the highest concentration of CRP. The footprints for HapR look similar. This is somewhat troubling as in these types of experiments one would like to observe a dose dependent change in the footprint correlating with more DNA occupancy.

For CRP we agree but are not concerned at all by this. The site is simply full occupied at the lowest protein concentration tested. Given that the footprint exactly coincides with a near consensus CRP site (which, when mutated, abolishes CRP binding in EMSAs, and regulation by CRP *in vivo*) all our results are perfectly consistent. Note that i) our only aim in this experiment was to determine the positions of CRP and HapR binding ii) our conclusions are independently backed up using gel shifts and by making promoter mutations. With respect to HapR, there are changes at the periphery of the main footprint.

1. Fig. 4e-Why does the transcriptional activation of *murQP* decrease with increasing concentrations of CRP? This is also seen in Fig. 5e.

In our experience, this often does happen when doing *in vitro* transcription assays (with CRP and many other activators). The anecdotal explanation is that, at higher concentrations, the regulator can start to bind the DNA non-specifically and so interfere with transcription.

13. The authors demonstrate in vitro that HapR requires binding of CRP to bind the murQP promoter. It would strengthen their model if they demonstrated this in vivo. To do this, the authors only need to repeat their ChIP-Seq experiment in a delta CRP mutant and the HapR signal at murQP would be lost. In fact, such an experiment would experimentally confirm which of the in vivo HapR binding sites are CRP dependent.

We agree, appreciate the comment, and do plan to do such experiments in the future as a wider assessment of interactions between transcription factors. However, doing this does have substantial time and resource implications that we cannot devote to the project at present. We feel that our overall conclusions, regarding co-operative interactions between HapR and CRP at PmurQP, are well supported by the data already provided. This also seems the overall opinion of the reviewers.

1. Fig. 5b-I am confused by the Venn diagram. The text states that "In all cases, the CRP and HapR targets were offset by 1 bp", but the diagram only shows 7 overlapping sites. The authors need to better describe these data.

We mean that, in all cases where sites overlap, sites are offset by 1 bp (i.e. we didn't find any sites

overlapping but offset by 2, 3 4 bp etc).

15. Line 287-288 and Fig. 5d-The authors state that HapR binds with less affinity to the CRP E55A mutant protein bound to DNA. There does seem to be a difference in the amount of shifted bands at the equivalent concentrations of HapR, but the difference is subtle. In order to make such a conclusion, the authors should show replication of the data and calculate the variability in the results. The authors should also use these data to determine the actual binding affinities of HapR to WT and the E55A mutant CRP, along with error or confidence intervals.

All of these experiments have been run multiple times and we are absolutely confident of the result. With respect to Figure 5d, this was done many times. We note that not all experiments were exact repeats. E.g. some of the first attempts had fewer HapR concentrations. Even so, the defect in HapR binding to the CRP E55A complex was always evident. The two gels to the left show the final two iterations of this experiment (these are exact repeats). The top image is that shown in Figure 5d. The lower image is an equivalent experiment run a day or so previously. Both clearly show a defect in HapR binding to the CRP E55A complex. We appreciate that our conclusion re these experiments is somewhat qualitative (i.e. that HapR binds the CRP E55A complex less readily) but this is not out of kilter with the vast majority of similar literature and our results are clearly reproducible.

1. Fig. 6a-It is odd that the locked low cell density mutants have such a growth defect in MurNAc, minimal glucose, and LB. To my knowledge, such a growth defect is not common with these strains. Perhaps this has to do with the specific growth conditions used here, but I can't find that information in the manuscript (it should be there). Furthermore, the growth rate of the luxO and hapR mutants appears to be similar up to the branch point (i.e. slope of the curve), but the lag phase of the luxO mutant is much longer. The authors need to address these issues in relationship to previous published literature and specify their growth conditions because the results are not consistent with their simple model described in Fig 6b.

This comment is a little difficult to pick apart as it covers several different issues. We'll try and answer these individually.

a) The unusual "biphasic growth curve with hapR and hapRluxO cells: We do not know why cells lacking hapR have a growth curve that appears biphasic. We can only assume that this is

due to some regulatory effect of HapR, distinct from the *murQP* locus. Despite the unusual shape of the growth curve, the data are consistent with our conclusions.

b) The extended lag phase of the *luxO* mutant in minimal media + MurNAC: We appreciate this comment and had considered possible explanations prior to submission. In the end, we left out this speculation but are happy to include it as part of our response. The extended lag phase might be expected if CRP/HapR regulation is largely critical for controlling the basal transcription of *murQP*. The locus is likely also regulated by the upstream repressor MurR (VC0204) as in *E. coli*. So, if derepression of MurR overwhelms the effect of HapR on *murQP*, we think you would expect that once the cells start growing on MurNAC, the growth rates are unchanged. But the extended lag is due to the fact that it took longer for those cells to achieve the critical threshold of intracellular MurNAC-6-P necessary to drive *murR* derepression. Obviously, we can not provide a definitive answer.

c) We have added further details regarding growth conditions to the methods section and the Figure 6a legend.

1. Fig. S6-The data to this point with murPQ suggested a model in which CRP binding then enabled HapR binding. But these EMSA suggest that both situations occur as in some cases, such as VCA0691, HapR binding promotes CRP binding. How does such a result fit with the structural model presented in Fig. 5?

This is to be expected and is fully consistent with the model. Cooperativity is a two-way street, and each protein will stabilise binding of the other. Clearly, it will not always be the case that the shared DNA site will have a higher affinity for CRP than HapR (as at *PmurQP*). Depending on the shared site sequence, expected that sometimes HapR will bind “first” and then stabilise binding of CRP.

18. Line 354-356-The HCD state of V. cholerae occurs in mid-exponential phase and several cell divisions occur before stationary phase and the cessation of growth, at least in normal laboratory conditions. Therefore, there is not support for the argument that QS is a mechanism to redirect cell wall components at HCD because cell wall synthesis is no longer needed.

We did not intent to suggest cell wall synthesis is not needed at all, rather that there is a reduced need. We made a slight change to the discussion to reflect this.

19. Line 357-360-Again, as stated in point 16, the statement that cells locked in the HCD are "defective for growth" is an oversimplification. The luxO mutants have a longer lag phase, but they actually outgrow the hapR mutants at higher cell densities and reach the maximum yield much faster.

In fairness, we do go on to specify that the defect is an extended lag phase. Also see our response above.

Reviewer #2 (Recommendations For The Authors):

Comments to improve the text

1. Line 103-106, line 130, line 136, etc. Details of the methods and the text directing to presentations of figures should be in the methods and/or figure legends with (Figure x) in citation after the statement. The sentences in lines indicated can be deleted from the results. Although several lines are noted specifically here, this comment should be applied throughout the entire results section.

We appreciate this comment but would prefer not to make this change (it seems mainly an issue of personal stylistic choice). It is sometimes helpful for the reader to include such

information as it avoids them having to cross reference between different parts of the manuscript.

1. Line 115. Recommend a paragraph between content on LuxO and HapR (before "Of the 32 peaks for HapR binding")

We agree and have made this change.

1. Line 138 and Figure 1a. I am not convinced this gel shows that VC1375 is activated by HapR. Is the arrow pointing to the wrong band? There does seem to be an induced band lower down.

We understand this comment as it is a little difficult to see the induced band. This is because this is a compressed area of the gel and the transcript is near to an additional band.

1. Line 147. Add the VC0206-VC0207 next to *murQP* (and the gene name *murQP* into Table 1).

We have added the gene name to the figure foot note. The text has been changed as requested.

1. Methods. It is essential for this paper to have detailed methods on the bacterial growth conditions. Referring to prior paper, bacteria were grown in LB (add composition...is this high salt LB often used for vibrios or low salt LB often used for *E. coli*). Growth is to "mid log". Please provide the OD at collection. Is mid log really considered "high density". Provide a reference regarding HapR activity at mid log to support the method. Could the earlier collection of bacteria account for missing known HapR regulated genes? In preparing the requested ζ , include growth conditions for other experiments in the legends.

Note that we have included a new supplementary table, rather than a Venn diagram. We have also added further details of growth conditions as mentioned above. Also not that, for the ChIP-seq, HapR and LuxO were expressed ectopically and so uncoupled from the switch between low and high cell density.

1. Content of Table 1, HapR Chip-seq peaks, needs to be closely double checked to the collected data as there seems to be some errors. Specifically, VC0880 and VC0882 listed under Chromosome I are most likely VCA0880 (*MakD*) and VCA0882 (*MakB*), both known HapR induced genes on Chromosome II with VCA0880 previously validated by EMSA. This notable error suggests the table may have other errors and thus requires a very detailed check to assure its accuracy.

We appreciate the attention to detail! We have double checked, thankfully this is not an error, the table is correct (even so, we have also checked all other entries in the table). As an aside, VCA0880 is one of the locations for which we see a weak HapR binding signal below our cut-off (included in the new Table S1). In cross checking between Table 1 and all other data in the paper we noticed that we had erroneously included assay data for VC0620 in Figure 2A. This was not one of our ChIP-seq targets but had been assayed at the same time several years ago. This datapoint, which wasn't related to any other part of the manuscript, has been removed.

If VCA0880 and VCA0882 are correctly placed on Chr. I, then add comment to text that the *Mak* toxin genomic island found on Chromosome II in N16961 is on Chr. I in E7946. (See recent references PMID: 30271941, 35435721, 36194176, 34799450).

See above, this is not an error.

1. Alternatively for both comments 8 & 9, are these problems of present/missing genes or misannotations the result of the annotation of E7946 gene names not aligning with gene names of N16961? (if so, in Table 1, please give the gene name as in E7946 but include a separate column with the N16961 name for cross study comparison)

See above and below, this is not an issue.

1. Line 126-127. Also regarding Table 1, please add a column with function gene annotation. For example, VC0916 needs to be identified as vpsU. If function is unknown, type unknown in the column. This will help validate the approach of selecting "HapR target promoters where adjacent coding sequence could be used to predict protein function."

We added an extra column to Table 1 in response to a separate reviewer request (TSS locations). This leaves no space for any additional columns. Instead, to accommodate the reviewer's request, we have added alternative gene names to the footnote.

Not following up on VCA0880 (promoter for the mak operon) is a sad missed opportunity here as it is one of the most strongly upregulated genes by HapR (PMC2677876)

As noted above, this was not an error and VCA0880 was not one of our 32 HapR targets. As such, we would not have followed this up.

1. Figure Legends. Add a unit to the bar graphs in Figure 1e (should be kb??) This has been corrected.
2. The yellow color text labels in figures 3c, 4a, 4c are difficult to read. Can you use an alternative darker color for CRP.

We have made this slightly darker (although to our eye it is easily reliable). We haven't changed the colour too much, for consistency with colour coding elsewhere.

1. Figure S3. Binding is misspelled. Add units to the x-axis

This has been corrected.

1. Figure S7. The text in this figure is too small to read. Figure could be enlarged to full page or text enlarged. Are these 4 the only other known regulated promoters? Could all the known alternative promoters linked to HapR be similarly probed?

We have increased the font size and included a new Table S1 for all previously proposed HapR sites.

1. Figure S8. Original images..are any of these the replicate gels (see public comment 6)

We have added a statement regarding reproducibility, and also note the internal reproducibility between different figures in our reviewer response. The gels in Figure S8 are full uncropped versions of those shown in the main figures.

Reviewer #3 (Recommendations For The Authors):

None

Whilst there were no specific recommendations from this reviewer, we have still responded to the public review and made changes if required.