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Maria L. Nellessen 04-18-2012

# Identifying genes controlled by the catabolite repressor protein, CRP, and the anti-activator protein, CytR, and their roles in the quorum sensing and natural competence pathways in *Vibrio cholerae*

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2012

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An abstract of a thesis submitted to the Faculty of Emory College of Arts and Sciences of Emory University in partial fulfillment of the requirements of the degree of Bachelor of Sciences with Honors

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ABSTRACT

Identifying genes controlled by the catabolite repressor protein, CRP, and the anti-activator protein, CytR, and their roles in the quorum sensing and natural competence pathways in *Vibrio cholerae*

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The bacterium, *Vibrio cholerae* is a natural inhabitant of brackish waters, but can also cause the diarrheal disease, cholera, when ingested by a human host. In the environment, *V. cholerae* often attaches to chitinous surfaces such as those found on crabs and copepods. In the presence of chitin, a *V. cholerae* cell-cell communications system called quorum sensing (QS) induces expression of genes permitting this pathogen to take up extracellular DNA. The purpose of this study is to define the genetic components and regulatory connections that link QS and chitin signaling to natural transformation, using molecular genetics and biochemical techniques. Natural transformation is one mechanism of horizontal gene transfer (HGT) that allows individual cells to incorporate foreign DNA fragments into their genome. Such a process may increase the evolutionary fitness of the bacterium because the incorporated DNA may encode beneficial genes such as those coding for antibiotic resistance. Recently, it has been shown that *V. cholerae* natural competence utilizes a molecular mechanism involving two regulators, CRP and CytR. The goal of the studies described below was to define the role that these two genes, other genetic components, and the environment play in the evolution of this waterborne pathogen.

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## INTRODUCTION

*Vibrio cholerae* is a Gram-negative bacterium that is the causative agent of cholera, a diarrheal disease that can be life-threatening without prompt and proper treatment. Upon ingestion of the bacteria in contaminated food or water, *V. cholerae* can survive passage through the stomach and gain access to the small intestine where it produces attachment factors and the cholera toxin (CT). CT is a classical A/B toxin that has been well characterized for its important role during cholera infection. The toxin recognizes and binds to receptors on intestinal epithelial cells, where it then gains access to the cytosol and interacts with the G-protein counterpart of adenylate cyclase. This initiates over production of cAMP and prostaglandins, which results in electrolyte and water loss via excessive diarrhea and vomiting (Spangler, 1992).

*V. cholerae,* however, is also a common inhabitant of brackish waters and can live planktonically or on the surface of chitinous zooplankton in complex communities known as biofilms (Huq A, 1983; Tamplin et al, 1990). It is believed that attachment to zooplankton may allow *V. cholerae* to remain suspended in water columns and serve as a vector for transmission into a host. Thus, the aquatic reservoir and all of its changing factors, including seasonal shifts in chitinous organism density, nutrient availability, and climate change play a role in the epidemiological patterns of this water-borne pathogen (Colwell, 1996; Lipp et al, 2002; Lobitz et al, 2000; Vezzulli et al, 2010). Biofilm formation on chitinous surfaces is a result of complex gene expression that contributes to the attachment of *V. cholerae* to a microcosm composed of exopolysaccharide (EPS)

matrices and other bacterial species. Control of the *vps* (*Vibrio* polysaccharide) genes associated with biofilm formation depends upon environmental conditions and signals that modulate regulatory networks in *V. cholerae* and other microorganisms (Donlan, 2002). One such environmental signaling system that regulates biofilm formation in *V. cholerae* is the process of cell-cell communication called quorum sensing (Hammer & Bassler, 2003).

## QUORUM SENSING

As in many other bacteria, *V. cholerae* populations have the ability to regulate gene expression based on cell density by producing, releasing, and then recognizing small diffusible molecules called autoinducers (AIs) in a social process described as quorum sensing (QS) (Fuqua et al, 1994; Ng & Bassler, 2009). *V. cholerae* produces two QS signal molecules; a *Vibrio*-specific autoinducer, CAI-I (produced by the CqsA synthase) that is only produced by members of the genus; and an inter-specifies autoinducer, AI-2 (produced by the LuxS synthase) that is also made by many other bacteria. Both signals transduce information into the same QS response pathway to regulate expression of numerous genes including those for biofilm formation and virulence (Chen et al, 2002; Hammer & Bassler, 2003; Higgins et al, 2007; Xavier & Bassler, 2005).

Numerous studies have defined the components of the QS regulatory network and based on these studies a model is proposed to understand how the QS system regulates genes in response to the changes in bacterial cell density (see Figure 1 and for review (Ng & Bassler, 2009)). When *V. cholerae* are at low

cell density (LCD), and AI levels are low, both AI receptors (CqsS for CAI-I, and LuxP/Q for AI-2) are unbound and act as histidine kinases, transferring phosphate, via LuxU, to a response regulator protein, LuxO. Phosphorylated LuxO (LuxO~P), together with the alternative sigma factor $\sigma^{54}$, acts as a DNA-binding response regulator, which binds to the promoter region and activates transcription of four quorum-regulatory RNAs (*qrr1-4*) (Lenz et al, 2004). These functionally redundant small RNAs (sRNAs) directly bind to the ribosome binding site of HapR mRNA, with the aid of the Hfq RNA chaperone, to post-transcriptionally prevent translation of HapR protein, which is the master regulator of the QS response pathway (Bardill et al, 2011). So at LCD, in the absence of HapR, the *vps* exopolysaccharide genes for biofilm formation are expressed and the *ctxAB* cholera toxin (CT) gene and the *tcp* pilus genes are also expressed. Production of the TCP and VPS attachment factors and CT toxin are thought to promote colonization and pathogenicity of the small intestine (Hammer & Bassler, 2003; Zhu et al, 2002).

When *V. cholerae* reaches high cell density (HCD), CAI-I and AI-2 bind to their cognate receptors, which reverses the flow of phosphate from LuxO, halting *qrr*1-4 production, and allowing translation of *hapR* (Bardill et al, 2011; Hammer & Bassler, 2007; Lenz et al, 2004; Rutherford et al, 2011; Svenningsen et al, 2009). HapR is a transcription factor that serves both to activate and repress gene expression for multiple targets. At HCD, when HapR is made due to the presence of AI-2 and CAI-1, the *vps, ctx,* and *tcp* genes are repressed (Miller et al, 2002; Zhu et al, 2002). It is proposed that negative regulation of these factors

at HCD promotes transmission of the bacterium out of the *in vivo* host and back into the environment where *V. cholerae* has the potential of becoming ingested by a new host or where it can survive in an aquatic niche (Hammer & Bassler, 2003; Miller et al, 2002; Zhu & Mekalanos, 2003; Zhu et al, 2002) This model of the *V. cholerae* QS system is depicted in Figure 1.

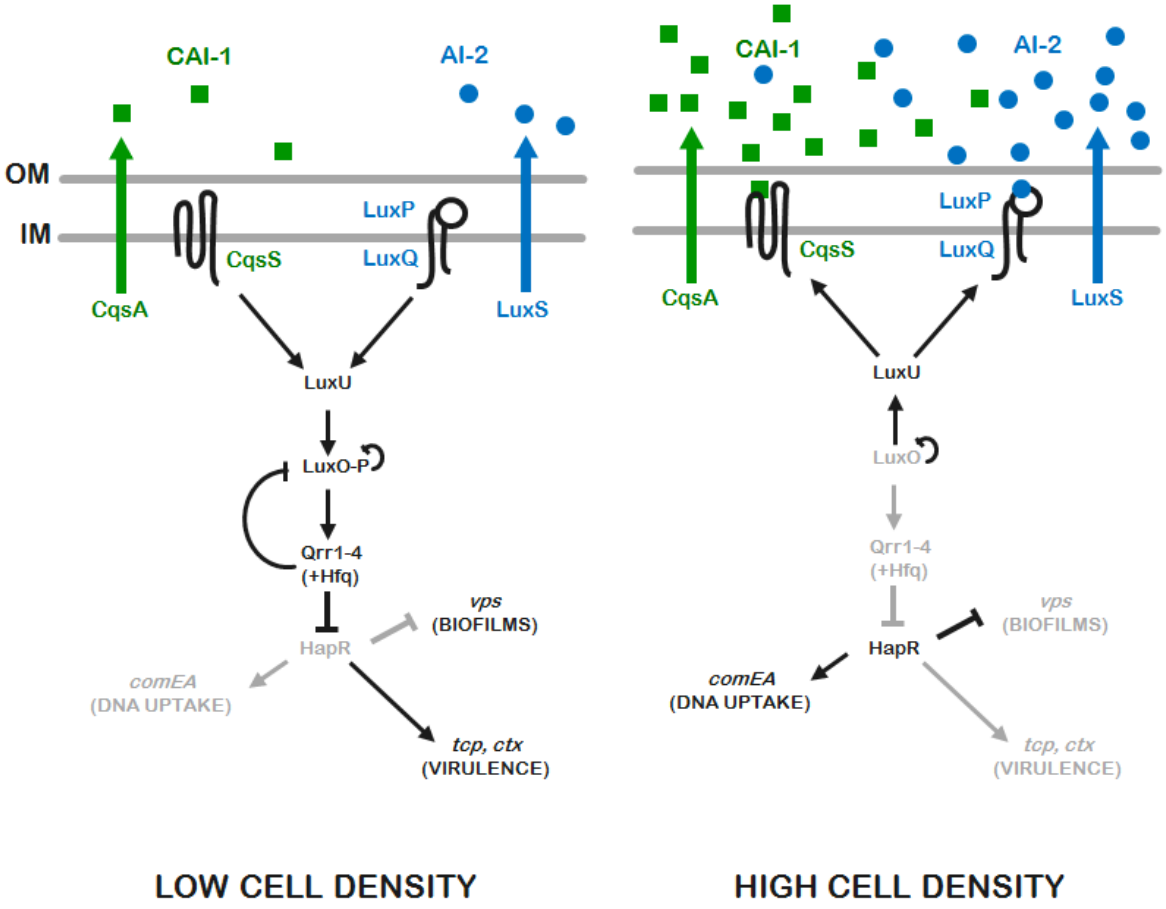


**Figure 1: QS model for *V. cholerae.* The defined regulatory pathway at LCD (left) and HCD (right).**

The *V. cholerae* QS pathway is fine-tuned through multiple feedback loops in order to distinctly facilitate transition between LCD and HCD states. In the related species, *V. harveyi*, which has a similar QS system, LuxO has been

shown to autorepress its own transcription. Specifically, LuxO protein recognizes and binds to a consensus sequence, TTGCA($N_3$)TGCAA (where N indicates an A or T), upstream of each of the four *qrr* genes. When phosphorylated, LuxO interacts with RNA polymerase (RNAP) to promote *qrr* transcription (Lenz et al, 2004). The promoter region of *qrr*1, in particular, lies proximal to the promoter region of *luxO*. When LuxO~P binds to the LuxO binding site to initiate *qrr*1 transcription (Figure 2), it also overlaps the -35 position of the *luxO* promoter region, thus repressing its own transcription (Figure 1, LuxO auto-repression) (Tu et al, 2010). The Qrr sRNAs in return also post-transcriptionally repress *luxO* by binding to and interfering with the ribosome binding site (RBS) in the 5' untranslated region (UTR) of *luxO* mRNA, thus preventing translation into LuxO protein (Figure 1, Qrr repression of LuxO) (Tu et al, 2010). The LuxO promoter region of *V. cholerae* is depicted in Figure 2.

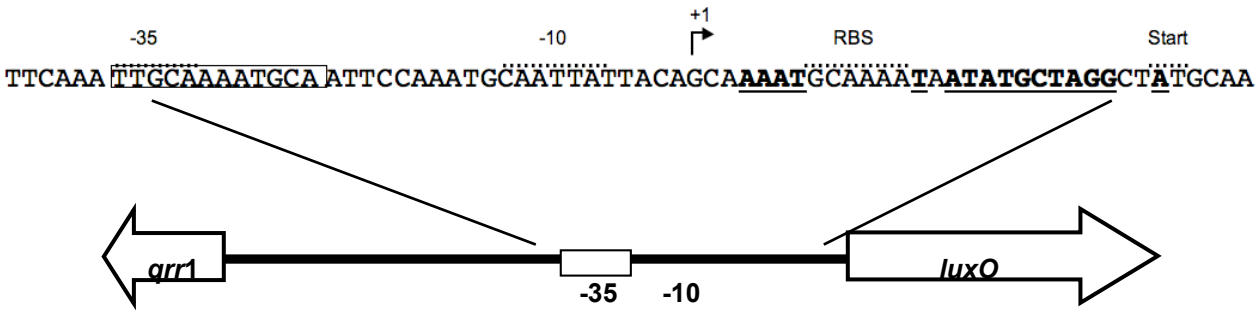


**Figure 2: The LuxO promoter region depicting transcriptional and translational autorepression. Highlighted in the *luxO* nucleotide sequence (above) is the -35 and -10 position depicted for RNAP binding, the +1 of transcription determined by 5' RACE, the RBS, and the ATG start codon for translation of LuxO protein. The boxed sequence is the LuxO binding site for *qrr*1 transcription. The underlined nucleotides in the UTR indicate predicted positions for Qrr base pairing. These positions relative to *luxO* and *qrr*1 on the chromosome are depicted below. Figure adopted from (Tu et al, 2010).**

## NATURAL COMPETENCE AND TRANSFORMATION

Recently, it has been shown that when grown to HCD, QS-proficient *V. cholerae* is also naturally competent to take up extracellular DNA (Meibom et al, 2005). Natural competence is a type of Horizontal Gene Transfer (HGT) that pertains to the ability of bacteria to utilize cellular components to take up exogenous DNA and incorporate the genetic material into their genome. Specifically, QS-proficient *V. cholerae* were shown by Meibom et al. to be naturally competent only in the presence of chitin, a polymer of $GlcNAc_n$ that can be used as a nutrient source in otherwise nutrient-limited marine environments (Meibom et al, 2005). It is proposed that a chitinous surface provides both a nutrient and a niche where bacteria can form biofilms with other marine microorganisms, thus giving *V. cholerae* ample opportunity to take up DNA (Blokesch & Schoolnik, 2007). This phenomenon is thought to allow *V. cholerae* and other *Vibrios* to communicate via QS AIs and transfer genetically beneficial traits, promoting rapid genomic assortment and contributing to the mosaic genome structure of *Vibrios*, as shown in a recent comparative genomics study (Antonova & Hammer, 2011; Chun et al, 2009)

In the presence of chitin, *V. cholerae* produces numerous chitinases to degrade chitin polymer into oligomeric subunits (Meibom et al, 2004). Binding of chitin oligosaccharide $GlcNAc_6$ to CBP (chitin binding protein) has been shown to initiate, via the ChiS histidine kinase, transcription of an Hfq-dependent sRNA, TfoR, that binds to and promotes translation of the regulatory protein TfoX (Meibom et al, 2005; Yamamoto et al, 2011). Tfox is an ortholog of Sxy, which is

a critical regulator of the early stages of natural competence in *Haemophilis influenzae* (Redfield et al, 2005). In *V. cholerae*, microarray analysis revealed that chitin, or the experimental induction of *tfoX* expression, not only upregulates a number of genes involved in chitin utilization, such as *chiA-1* and *chiA-2* (extracellular chitinases) and *vc0972* (a putative chitoporin), but also upregulates natural competence genes, such as *comEA* (a DNA receptor involved in natural competence in *Bacillus subtilis* (Provvedi & Dubnau, 1999)), and *pilA* (the natural competence pseudopilus) (Meibom et al, 2004). Thus, natural transformation in *V. cholerae* occurs only in response to two sets of extracellular signal molecules: QS AIs and chitin (Antonova & Hammer, 2011; Blokesch & Schoolnik, 2007; Meibom et al, 2005).

## CytR, and anti-activator protein

TfoX and HapR are both required for maximal *comEA* transcription and for natural transformation, because a deletion of either gene prevents *comEA* expression and DNA uptake (Meibom et al, 2005). However, a direct link between TfoX, HapR, and *comEA*, remains unclear, as neither regulatory protein has been shown to directly interact with the promoter region of the *comEA* gene. Recently, a genetic screen was initiated in the Hammer lab utilizing Tn5 transposon mutagenesis of a *V. cholerae* strain carrying a plasmid-borne transcriptional fusion of the *comEA* gene to the luciferase operon (*comEA-lux*). The *comEA* gene was chosen for expression monitoring due to its role in natural competence (Provvedi & Dubnau, 1999). Specifically, the screen was performed

in a *V. cholerae* strain that constitutively expressed *tfoX*, independent of chitin (*tfoX**), and also constitutively expressed HapR, independent of AIs because of a *luxO* gene deletion that "locked" the strain at HCD (Δ*luxO*). This strain expresses *comEA-lux* at high levels ($Lux^+$). In the screen, ~20,000 Tn5 mutants were arrayed with a Genetix $QPix2^{xt}$ colony picker to microtiter plates, and screened with a BioTek® multimode plate reader to identify candidates with defective *comEA*-lux expression ($Lux^-$). One candidate was identified and standard methods were used to map the Tn5 insertion (Larsen et al, 2002). The gene with the Tn5 insertion was identified as *vc2677*, which was annotated in the *V. cholerae* database as a LacI family repressor protein.

A literature search revealed that the product of *vc2677* was identified as CytR in 2002 and shown to be a repressor of biofilm formation in *V. cholerae* (Haugo & Watnick, 2002). A 2005 microarray analysis also included *vc2677* among a list of TfoX induced genes. In *Escherichia coli*, CytR has been studied extensively and encodes an anti-activator of transcription that plays a role in nucleoside metabolism (Meibom et al, 2005; Valentin-Hansen et al, 1996). PhD student Elena Antonova constructed a Δ*cytR* deletion mutant of *V. cholerae* and showed that compared to the isogenic $cytR^+$ strain, this mutant had significantly lower expression of *comEA* and did not take up DNA, while a Δ*cytR* mutant complemented with a functional copy of *cytR* at the *lacZ* locus had restored normal *comEA* levels and was naturally competent to take up exogenous DNA. Refer to Figure 3 for these results (Antonova, Bernardy & Hammer; unpublished results).

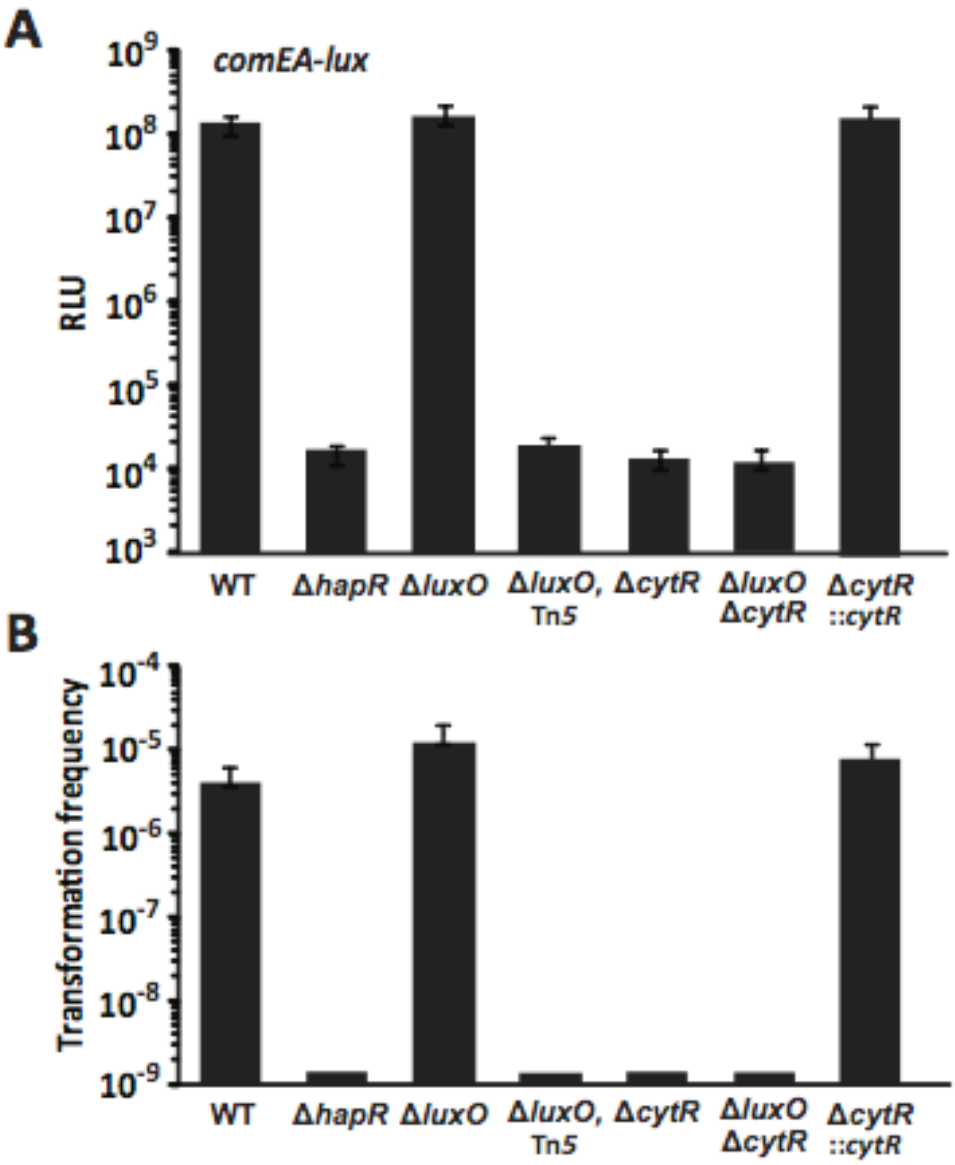


**Figure 3: TfoX and HapR-dependant *comEA* expression and natural transformation require CytR. A) *comEA* expression in strains with constitutive *tfoX* expression. RLU is defined as *lux* counts $min^{-1}mL^{-1}/OD_{600}$. Wild type (WT) *V. cholerae* expresses *comEA-lux* to high levels. Transcription of a *comEA*-lux reporter fusion is diminished in a strain locked at LCD (Δ*hapR*), and expression levels are similar to WT in a mutant strain locked at HCD (Δ*luxO*). Transcription also was diminished in both the strain with the Tn5 insertion and in a Δ*cytR* mutant. Complementation of *cytR* at the *lacZ* site (Δ*cytR::cytR*), restores expression. B) Efficient DNA uptake (transformation frequency) was calculated by the percent of CFUs able to take up extracellular KanR-marked DNA. Similar patterns for *comEA-lux* expression and transformation frequency are observed.**

Based on these results a new regulatory model for *comEA* expression is proposed as depicted in Figure 4, and discussed in greater detail below.

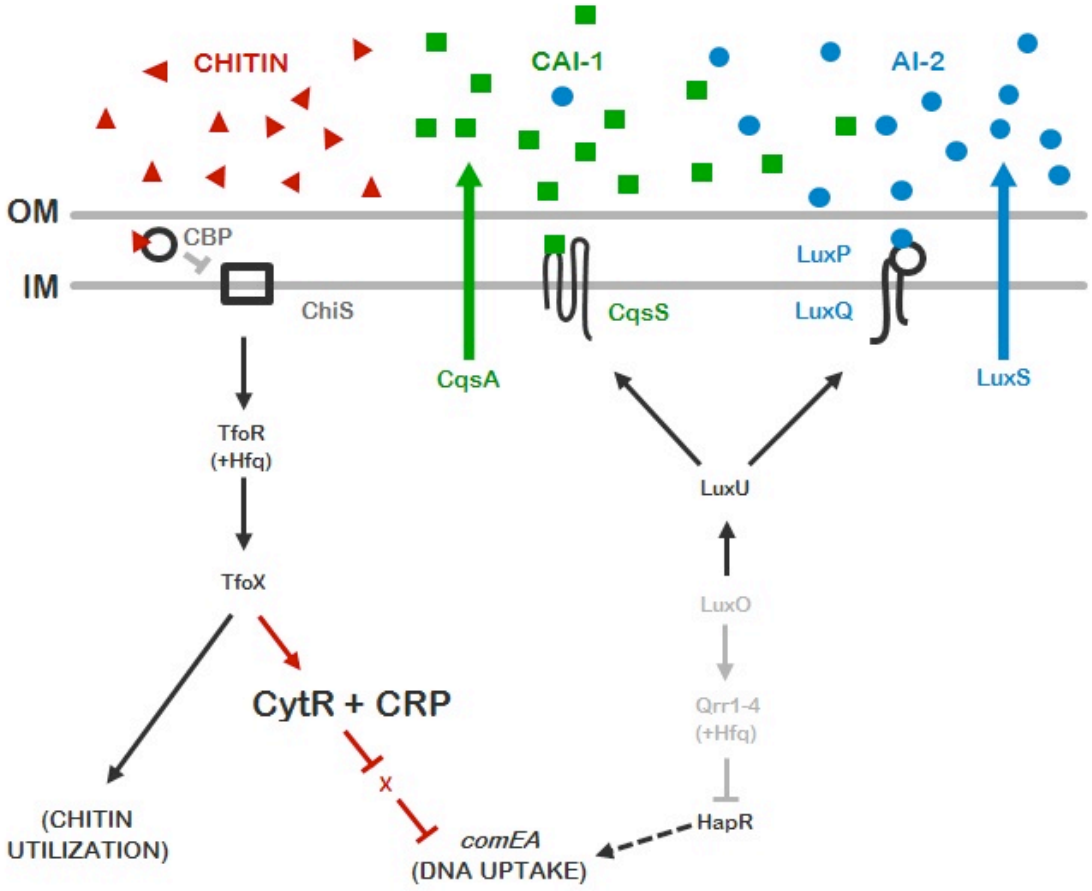


**Figure 4: Predicted role of CytR and CRP in *comEA* expression and DNA uptake.**

CytR structure and function have been extensively described in *E. coli* (Valentin-Hansen et al, 1996). In *E. coli*, CytR acts in tandem with the cAMP receptor protein (CRP), also known as CAP (for Catabolite Activator Protein). In the absence of preferred carbon sources (like glucose), *E. coli* CRP activates over 100 genes involved in metabolism by promoting the binding of RNAP upstream of the start of transcription (Busby & Ebright, 1999; Valentin-Hansen et

al, 1996). Although some CRP-activated genes in *E. coli* contain a single binding motif, a subset of CRP-activated genes contains two CRP binding motifs upstream of the promoter region. CRP binds to the consensus sequence TGTGA-($N_6$)-TCACA as a dimer (Busby & Ebright, 1999; Kolb et al, 1993; Parkinson et al, 1996). For the *E. coli* DNA binding motif of CRP, refer to Figure 5 below.

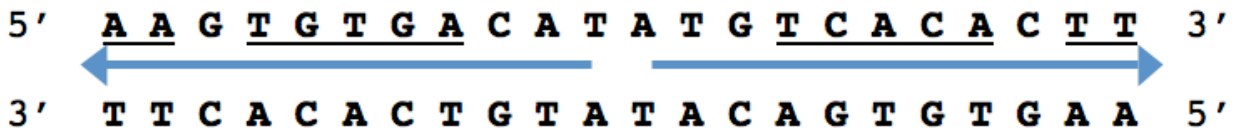


**Figure 5: DNA binding motif for a single CRP dimer. Underlined nucleotides indicate highly conserved bases indentified in promoters of known CRP-regulated genes in *E. coli*. Each blue arrow indicates a DNA binding motif for one monomer of the CRP dimer.**

In *E. coli,* promoters where two CRP binding motifs are identified, each motif is separated by ~50 nucleotides. When each CRP dimer complex is bound to the DNA upstream of a gene, each associates with a subunit of RNA polymerase (RNAP), activating transcription of the target gene (Figure 6A). However, for a particular set of so called “class III” CRP-activated genes, when CytR is present, this regulator competes with RNAP by binding within the ~50 nucleotide region between the two CRP dimer complexes, associating with both CRP proteins, and therefore preventing transcription (Busby & Ebright, 1999; Kallipolitis et al, 1997). Thus, CytR is defined as an “anti-activator” (Figure 6B).

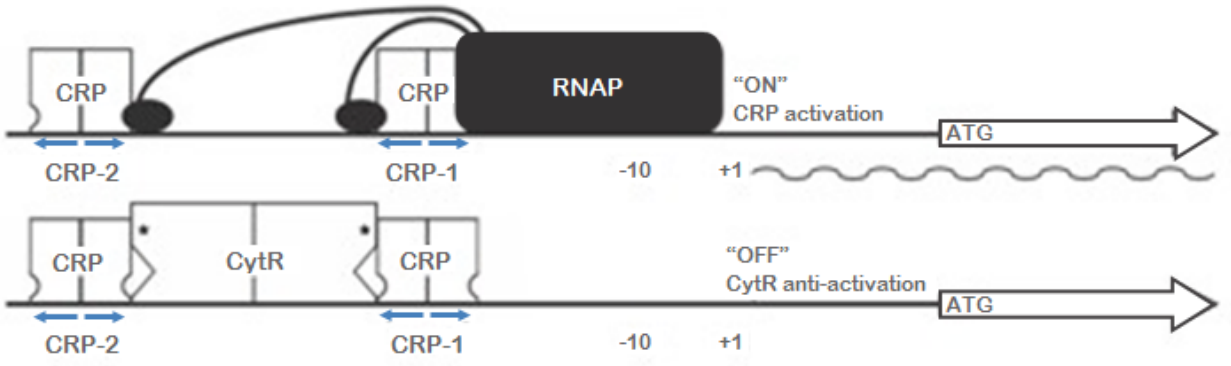


**Figure 6: Transcription activation of a promoter by CRP (above) by interaction of CRP with the two α-C-terminal domains of RNA polymerase (RNAP). Anti-activation of the same promoter by CytR-CRP interactions (below). The blue arrows characterize each CRP dimer that is bound to the CRP binding site (CRP-1 and CRP-2)**

In *E. coli*, it has been shown that CytR associates with CRP via protein-protein interactions, but contains a poor DNA binding domain (Kallipolitis et al, 1997). In known *E. coli* genes regulated by CRP-CytR anti-activation, such as *deoP2*, the CRP binding motifs in the DNA are well conserved, but the intervening CytR operator binding site is degenerate. Indeed, scrambling the DNA sequence between the two CRP sites of the *deoP2* gene in *E. coli* does not prevent CRP-CytR anti-activation (Rasmussen et al, 1993). Thus, CRP-CytR protein-protein interactions, and not binding of CytR to DNA, per se, are required for CytR-dependent anti-activation.

Protein alignments show that the *V. cholerae* and *E. coli* CytR and CRP proteins share 81% and 95% identities, respectively (Skorupski & Taylor, 1997). Importantly, the critical amino acids required for CytR-CRP protein-protein interactions in *E. coli* are present in *V. cholerae*, suggesting that CytR in *V. cholerae* also serves as a negative regulator, or anti-activator, of CRP-activated

genes (Figure 7). Specifically, in *V. cholerae,* the amino acids L161, F165, and R203, are predicted to be in a patch on CytR that interacts with H18, V109, and P111 of CRP. Indeed, PhD student Antonova has shown that a single amino acid substitution (L161A) in *V. cholerae* CytR prevents CytR control, consistent with an anti-activation mechanism working in *V. cholerae,* like in *E. coli*, via CytR-CRP protein-protein interaction (Antonova, Bernardy & Hammer; unpublished results)


**Figure 7: A) Protein sequence alignment for CytR in *V. cholerae* (above) and *E. coli* (below). The boxed amino acids are conserved and were shown in *E. coli* to facilitate necessary protein-protein interactions with CRP. B) Protein sequence alignment for CRP in *V. cholerae* (above) and *E. coli* (below). The red amino acids are conserved and were shown in *E. coli* to facilitate necessary protein-protein interactions with CytR**

## BIOINFORMATICS PREDICTION OF CYTR-REGULATED GENES IN *V. CHOLERAE*

We sought to identify genes controlled by CRP-CytR interaction in *V. cholerae* to understand the genes and regulatory connection that link QS and chitin to natural competence in this organism. Based on the understanding that in *E. coli* the DNA binding motif for CRP, but not for CytR, is well conserved, collaborator Vani Rajan, from the lab of Dr. Mark Bordovsky, used bioinformatics methods to scan the *V. cholerae* genome to identify genes with two putative CRP binding motifs upstream of the promoter region and separated by ~50 nucleotides. A set of ~100 *V. cholerae* genes was predicted to be under control of CytR anti-activation and involvement in the QS and natural competence pathway (Rajan & Bordovsky, unpublished results). Three of these candidate genes are depicted in Figure 8 below and are the subject of this study.

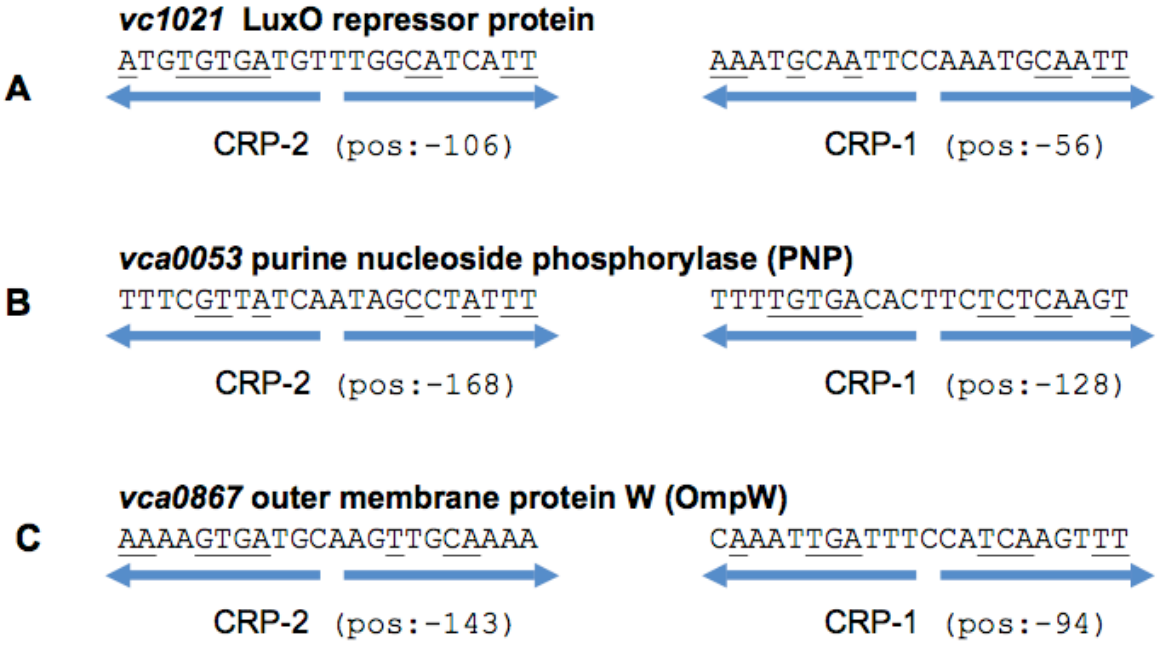

**Figure 8: Candidate *V. cholerae* genes predicted to be controlled by CytR anti-activation, including *vc1021* (A), *vca0053* (B), and *vca0867* (C). Under each gene annotation are both CRP dimer motifs (that flank a 40-50 nucleotide region). Nucleotides that are underlined share the CRP binding motif described in Figure 3. The two positions (pos.) indicated for each gene refer to the distance of the midpoint of the motif relative to the ATG start codon of the gene. Each blue arrow indicates a DNA binding motif for one monomer of the CRP dimer.**

Ninety-six annotated candidate genes were identified that have two putative CRP binding motifs, separated by 40-60 nucleotides. Ten genes were annotated in the *V. cholerae* database as encoding regulatory proteins. In particular, the QS response regulator gene, *luxO* (*vc1021*), was identified as a potential gene both activated by CRP and anti-activated by CytR (Figure 8). Consistent with a potential role of CRP in *luxO* regulation, a Δ*crp* mutant was shown previously by the Benitez group to be altered in QS, which suggested a role for catabolite repression of QS. In this prior study, however, the mechanism by which CRP acts on QS was not determined, and a role of CytR was not described (Liang et al, 2007). Since it has been shown that the QS pathway is involved in natural transformation, the QS response regulator, LuxO, was chosen as one of three target genes to test for CRP activation and CytR anti-activation in this study.

Fifty-eight additional candidate genes were identified as potentially under CRP and CytR control and are not annotated as regulators, instead are the first gene in an operon or a stand-alone gene. Two of these genes, *vca0053* (PNP) and *vca0867* (OmpW) were previously shown by microarray analysis to exhibit lower expression in a *V. cholerae* Δ*crp* mutant relative to the isogenic WT strain (Liang et al, 2007). This suggests these genes are directly CRP activated, and perhaps CytR anti-activated, although again, the Benitez group did not explore

mechanism of CRP control of *vca0053* and *vca0867.* So too, these prior microarray studies were unable to address the molecular mechanism of CRP regulation, as arrays are not able to identify genes that might be directly bound at their promoters by CytR and CRP. However, our bioinformatics predictions suggest that v*ca0053* and *vca0867* may be directly controlled by CytR/CRP anti-activation. The *vca0053* gene encodes for a type of purine nucleoside phosphorylase (PNP) that are ubiquitous in both prokaryotes and eukaryotes and involved in the purine nucleotide salvage pathway (Bzowska et al, 2000). Because CytR in *E. coli* participates in regulation of nucleotide metabolism genes, this served as an interesting gene to explore in this study. The *vca0867* gene encodes a small outer membrane protein (OMP) common in Gram-negative bacteria that is thought to play a role in the transport of small molecules in and out of the cell due its β-barrel structure with a hydrophobic core (Hong et al, 2006). *V. cholerae ompW* has been sequenced and has been a target for novel drug and vaccine therapies for the treatment of cholera due to its immunogenic properties (Das et al, 1998; Jalajakumari & Manning, 1990). The *ompW* gene was chosen as the third candidate gene to test for CRP and CytR control due to prior evidence suggesting that indeed this gene is under CRP regulation (Liang et al, 2007).

Each of the three genes was tested as described below to determine whether each is indeed CRP regulated, and more importantly if any of the three are also CytR anti-activated. This study will aide in developing methods for screening for intermediate genes involved in the natural competence pathway

(Figure 4), as well as help describe the role of CRP, and possibly CytR in the well-defined QS pathway.

# MATERIALS AND METHODS

## BACTERIAL STRAINS, PLASMIDS, AND CULTURE CONDITIONS

*V. cholerae* El Tor wild-type, its relative derivatives, and plasmid used in this study are listed in Table 1. Primers for target gene amplification were constructed in VectorNTI (Invitrogen) and are listed in Table 2. For experiments measuring expression of luciferase gene fusions, *V. cholerae* strains were incubated at 37ºC on Luria-Bertani (LB) agar, and in LB broth while agitated at 250 rpm. Artificial seawater (ASW; Instant Ocean) and crab shells were used for genetic manipulations where specifically indicated. The antibiotics (Fischer BioReagents) ampicillin (Amp), polymyxin B (Pb), chloramphenicol (Cm), kanamycin (Kan), and streptomycin (Str) were used in concentrations of 10, 10, 10, 100, 5000 μg $mL^{-1}$, respectively, where required.

## DNA MANIPULATIONS

Standard methods were followed for all DNA manipulations (Sambrook & Russell, 2001). *Spe*I (Promega), *Bam*HI (Promega), and *Bgl*II (New England Biolabs) restriction endonucleases, T4 DNA ligase (Promega) and Phusion® High Fidelity DNA Polymerase (New England Biolabs) were used for cloning. Colony PCR and other confirmatory PCR reactions were performed with GoTaq DNA Polymerase (Promega).

**Construction of luciferase-based transcriptional reporters.** Primers were constructed for amplification of a portion of the gene regions of *vc1021* (LuxO), *vca0053* (PNP), and *vca0867* (OmpW) from WT *V. cholerae*. Using the

known gene sequences, as a result of *V. cholerae* genome sequencing efforts (Heidelberg et al, 2000), primers were developed in VectorNTI (Invitrogen) that could be utilized to amplify a ~400 bp region (~200 bp upstream of the ATG and ~200 bp coding region) encoding the entire promoter region and a portion of the gene. For a depiction of the each gene locus, refer to Figure 9.

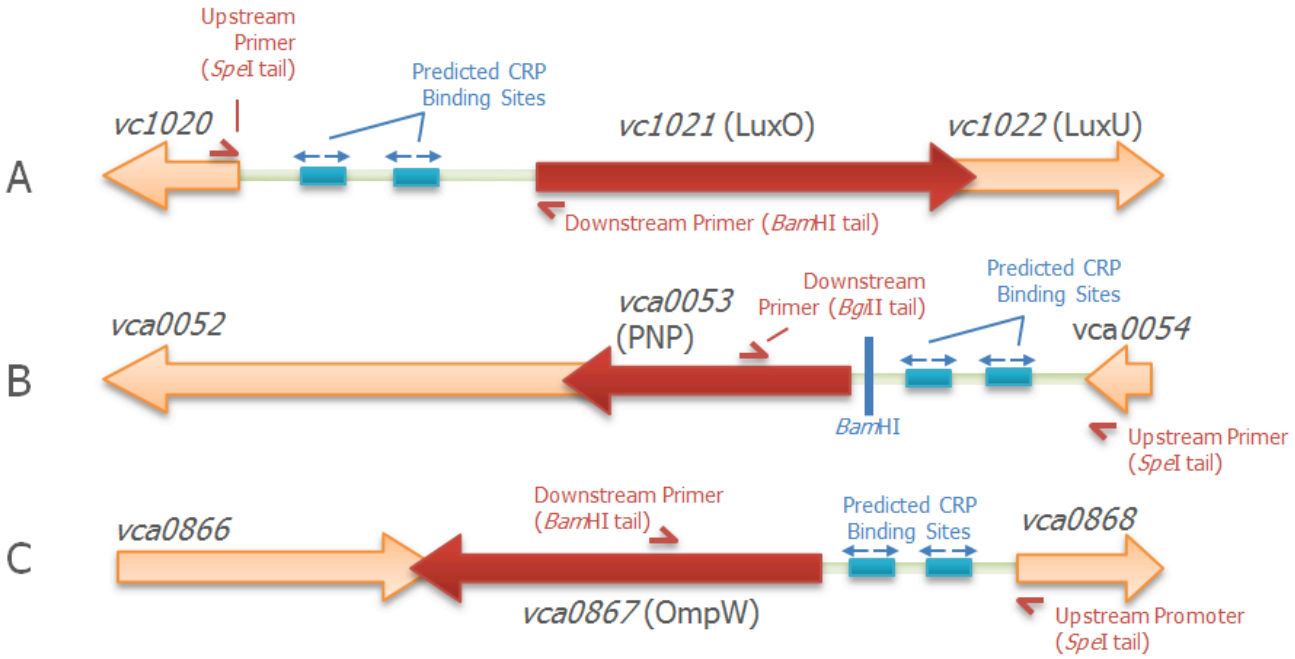


**Figure 9: Gene locus for *vc1021* (A), *vca0053* (B), *vca0867* (C). Each target gene is highlighted in red. Predicted CRP binding sites, location of designed primers with restriction enzyme tails for plasmid insertion, and relevant restriction sites are depicted. The primers for each gene were designed to amplify ~400 bp DNA fragments.**

*Spe*I and *Bam*HI restriction sites were added to the primers for ligation of each PCR product into the *Spe*I/*Bam*HI restricted pBBR*lux* vector that carries chloramphenicol (Cm) selection (described in (Lenz et al, 2004)). The gene *vca0053* contained a *Bam*HI restriction site in the desired amplification region, therefore, a *Bgl*II restriction tail was added in place of a *Bam*HI tail because

these *Bgl*II and *Bam*HI restriction endonucleases have compatible cohesive ends (New England Biolabs Technical Reference). Figure 10 depicts the pBBR*lux*-cloning vector with the *Bam*HI and *Spe*I restriction sites highlighted.

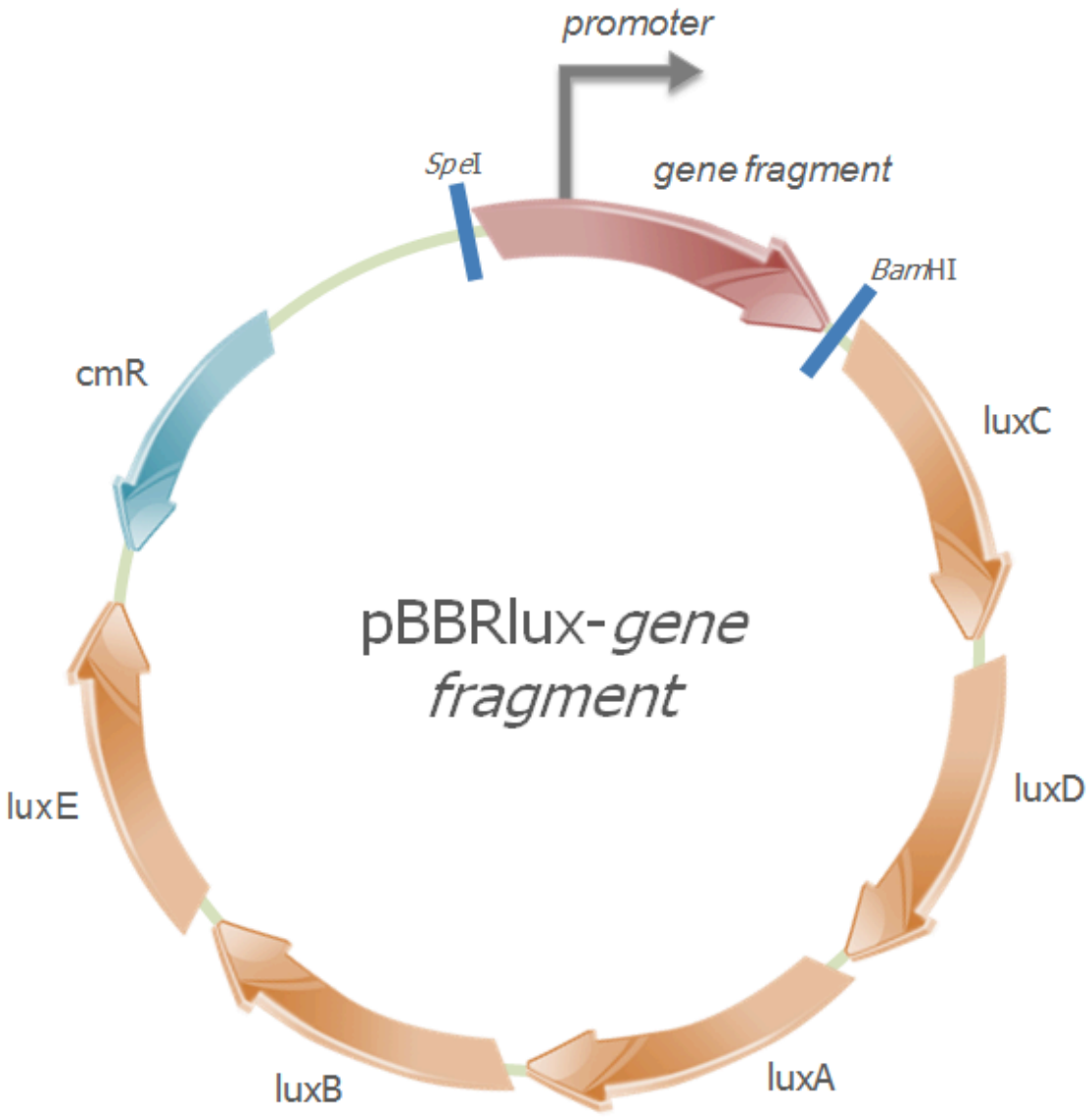


**Figure 10: Plasmid pBBR*lux* with site for insertion highlighted. The inserted *gene* with promoter region is depicted between the native *Spe*I and *Bam*HI restriction sites of the plasmid. The plasmid codes the luciferase operon (*luxCDABE*), as well as a chloramphenicol resistance gene (cmR) used for selection and plasmid maintenance.**

Genomic DNA (gDNA) was isolated from a culture of WT *V. cholerae* strain C6706 using a ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research). The designed primers (Table 2) were used to PCR amplify the target gene regions using WT gDNA as a template. The PCR products were applied to agarose gel electrophoresis (AGE) with 1% agarose in tris acetate EDTA (TAE) buffer at 95V. Ethidium bromide (EtBr) was added to the gel for sample visualization under UV light (365 nm). Each gene portion was excised from the gel and the DNA was recovered using a Zymoclean™ Gel DNA Recovery Kit.

The pBBR*lux* cloning vector was purified from an overnight culture of a host strain using a QIAprep® Miniprep Kit (Qiagen). Both the plasmid and the target genes were prepared for use in a restriction digest. Briefly, both restriction enzymes (*Spe*I and *Bam*HI for pBBR*lux*, *vc1021* (LuxO)*, vca0867* (OmpW); *Spe*I and *Bgl*II for *vca0053* (PNP)) were added to the DNA samples with the appropriate buffer according to manufacturer recommendations. The mixture was incubated at 37°C for at least 2 hours. The samples were applied to AGE and were recovered with the Zymoclean™ Gel DNA Recovery Kit. Each gene region was ligated to the pBBR*lux* cloning vector in a 3:1 mass ratio with T4 DNA ligase and was incubated at 16°C for 24 hours.

Each ligation mixture was electroporated into *E. coli* S17λpir cells. Briefly 1-4μL of each ligation mixture was added to 35μL of electro-competent cells in an electroporation chamber. The mixture was pulsed at 2.5kV with a GenePulser Xcell™ (Bio-Rad). The pulsed cells were then quickly recovered with 1mL LB and incubated at 37°C for 2 hours. Each culture was then plated on $LB_{cm}$ to select for

cells that acquired a pBBR*lux* plasmid. Colony PCR was performed to identify colonies with the correct gene insertion. Positive candidates were grown in $LB_{cm}$ and the plasmid was recovered using the QIAprep® Miniprep Kit (Qiagen). As an additional method for confirmation of gene insertion, a DNA digest was performed for the *luxO-lux* and *vca0867-lux* plasmids using the same restriction digest procedure mentioned previously. AGE was performed to verify a band at ~12 Kb (cloning vector) and the ~400 bp gene insertion. Candidate plasmids with insertions were sequenced and results were analyzed in VectorNTI (Invitrogen).

**Construction of relative isogenic strains.** As this study aimed to define the role of CytR in target gene expression, genetic manipulations of various strain backgrounds were performed to control expression of *cytR* utilizing our knowledge of the described chitin-induced pathway (Figure 4). To prevent the necessity to naturally induce TfoX, (and therefore CytR) via chitin-induction, the native *tfoX* promoter on the chromosome was replaced with the strong $P_{tac}$ promoter via allelic exchange with a pKAS32-based allelic exchange vector containing the desired insertion (Skorupski & Taylor, 1996). The $P_{tac}$ promoter is repressed by the product of the *lacI* gene and is therefore constitutively expressed in the *V. cholerae* strain used, which does not encode a functional *lacI* gene. As described in the Introduction, strains harboring this constructed *tfoX* allele are referred to as *tfoX**. Briefly, an *E. coli* S17λpir donor (Str and Pb sensitive) containing the pKAS-$P_{tac}$-*tfoX* plasmid (pEA291 in Table 1) was incubated with the desired *V. cholerae* recipient (Str and Pb resistant) to allow

conjugation of the allelic exchange vector. *V. cholerae* transconjugants that acquired the plasmid were selected for on $LB_{PB+amp}$ and colonies were restreaked on $LB_{amp}$. Single colonies were restreaked to $LB_{str}$ and were tested for $P_{Tac}$-*tfoX* insertion via colony PCR.

The pKAS32 allelic exchange system was also used to create a Δ*cytR* Δ*crp* double mutant and Δ*cytR* Δ*crp tfox** triple mutant. The procedure was performed as described above by conjugating pKAS-Δ*cytR* (pEA406 in Table 1) with both Δ*crp*::KanR and Δ*crp*::KanR *tfox** (EA577 and EA601, respectively) mutants. Colony PCR was used to verify gene deletion.

As an alternative to utilizing the described allelic exchange vector to create a *ΔluxO Δcrp* (MN187) double mutant and a *Δlux OΔcrp tfox** triple mutant (MN189), *crp* was deleted from the genome of both *ΔluxO* (SLS349) and *ΔluxO tfox** (EA281) by exploiting the ability of *V. cholerae* to undergo natural transformation. A plasmid containing a Kan marker that replaces the native *crp* gene, pKAS-Δcrp*::Kan* (pEA548) was isolated using a QIAprep® Miniprep Kit (Qiagen). Cultures of *ΔluxO* and *ΔluxO, ptac-tfoX*$^{chr}$ mutants were grown to exponential phase ($OD_{600}$ 0.3). The culture was washed in ASW (Instant Ocean) and grown to an $OD_{600}$ 0.15. Falcon® 12 well plates (Becton Dickinson) containing sterile crab shell fragments were inoculated with 2 mL/well of each culture and were incubated at 30ºC for 24 hours. The ASW was then aspirated from the wells and 2mL of fresh ASW was added to the cultures. Approximately 75 ng of the previously isolated plasmid (pEA548) was added to the media and the plate was incubated at 30ºC for 24 hours to allow for DNA uptake. The

culture and crab shell was then moved to a Falcon® tube and vortexed to removed cells that adhered to the chitin. The cells were washed and plated on $LB_{kan}$ to select for colonies that were able to take up the exogenous DNA containing the *kanR* gene. Individual colonies were screened via colony PCR to identify mutants where the *crp* coding sequence had been replaced by the *kanR* gene.

## BIOLUMINESCENCE ASSAYS

*E. coli* S17λpir donors containing the desired reporter plasmids were conjugated with *V. cholerae* recipients. *V. cholerae* strains carrying the *lux*-based reporter plasmid (cmR) were selected for on $TCBS_{cm}$ (Difco). TCBS (thiosulphate-citrate-bile salts-sucrose) is a selective and differential media because it selects for *Vibrios* and differentiates *V. cholerae* by characteristic yellow colonies (West et al, 1982). Single colonies were restreaked on $LB_{cm}$.

*V. cholerae* bioluminescence levels were assayed as previously described (Miller et al, 2002; Zhu et al, 2002). *V. cholerae* transconjugants containing the *lux*-based reporter plasmid were grown in $LB_{cm}$ at 37ºC overnight. The culture was diluted 1:1000 into fresh $LB_{cm}$ medium and incubated for approximately 8 hours. Appropriate dilutions were made to measure bioluminescence levels in a Wallace model 1409 liquid scintillation counter as previously described (Hammer & Bassler, 2007). The optical density ($OD_{600}$) of each culture was measured with a spectrophotometer. For each strain, Relative Light Units (RLU) was calculated as counts $min^{-1}$ $mL^{-1}$/ $OD_{600}$. End-point readings were measured in triplicate and

a one-tailed T-test with single variance was performed to compare expression levels in mutant backgrounds to the isogenic WT strain, as well as to compare each strain (*tfoX*⁻) to its *tfoX** counterpart.

**Table 1.** Bacterial strains and plasmids used in this study

| Strains | Genotype or description | Reference |
|---|---|---|
| *V. cholerae* strains | | |
| C6706str | El Tor biotype, O1; $HapR^+$ | (Thelin & Taylor, 1996) |
| EA305 | WT *tfoX** | (Antonova, Bernardy & Hammer, unpublished data) |
| SLS349 | Δ*luxO* | (Waters et al, 2008) |
| EA281 | Δ*luxO tfoX** | (Antonova, Bernardy & Hammer, unpublished data) |
| SLS340 | *LuxO D47E* | Lab collection |
| EA303 | *LuxO D47E tfoX** | This study |
| SLS456 | Δ*qrr1,* Δ*qrr2,* Δ*qrr3,* Δ*qrr4* | Lab collection |
| MN193 | Δ*qrr1,* Δ*qrr2,* Δ*qrr3,* Δ*qrr4 tfoX** | This study |
| BH1543 | Δ*hapR* | (Antonova & Hammer, 2011) |
| EA307 | Δ*hapR tfoX** | (Antonova, Bernardy & Hammer, unpublished data) |
| EA408 | Δ*cytR* | (Antonova, Bernardy & Hammer, unpublished data) |
| EA410 | Δ*cytR tfoX** | (Antonova, Bernardy & Hammer, unpublished data) |
| EA415 | Δ*luxO,* Δ*cytR* | This study |
| EA636 | Δ*luxO,* Δ*cytR tfoX** | This study |
| EA517 | Δ*cytR,* Δ*lacZ*::*CytR tfoX** | (Antonova, Bernardy & Hammer, unpublished data) |
| EA577 | Δ*crp*::KanR | (Antonova, Bernardy & Hammer, unpublished data) |
| EA601 | Δ*crp*::KanR *tfoX** | (Antonova, Bernardy & Hammer, unpublished data) |
| MN187 | Δ*luxO,* Δ*crp*::KanR | This study |
| MN189 | Δ*luxO,* Δ*crp*::KanR *tfoX** | This study |
| MN171 | Δ*cytR,* Δ*crp*::KanR | This study |
| MN173 | Δ*cytR,* Δ*crp*::KanR *tfoX** | This study |

| Plasmids | Features | Reference |
|---|---|---|
| pBBR*lux* | Cloning vector, $Cm^R$ | (Lenz et al, 2004) |
| pKAS32 | Cloning vector, $Amp^R$ | (Skorupski & Taylor, 1996) |
| pEA291 | pKAS-$P_{tac}$-*tfoX*, $Amp^R$ | (Antonova, Bernardy & Hammer, unpublished data) |
| pEA406 | pKAS-Δ*cytR*, $Amp^R$ | (Antonova & Hammer, 2011) |
| pEA548 | pKAS-Δ*crp::Kan* | This study |
| pMN105 | pBBR*lux-luxO*, $Cm^R$ | This study |
| pMN203 | pBBR*lux-vca0867*, $Cm^R$ | This study |
| pMN201 | pBBR*lux-vca0053*, $Cm^R$ | This study |

**Table 2.** Primers used in the study (noted restriction sites are underlined)

| Primers | Sequence | Description |
|---|---|---|
| GT704 | 5′ AAACTAGTCAGGCGCCACTGAGCAAGTGTTTAGG 3′ | LuxO promoter region amplification—contains *Spe*I restriction site tail |
| GT705 | 5′ AAGGATCCGGATTAAGTCAGGCTCGCGACGGCC 3′ | LuxO promoter region amplification—contains *Bam*HI restriction site tail |
| GT740 | 5′ AAACTAGTCCAACCCCACCAACCAAACGATTAGCTC 3′ | *vca0053* promoter region amplification—contains *Spe*I restriction site tail |
| GT741 | 5′ CCAGATCTGCAAGAAGGGATGCCCATGCC 3′ | *vca0053* promoter region amplification—contains *Bgl*II restriction site tail |
| GT742 | 5′ AAACTAGTCGTCATGCTCGCACTAGAAGAATG 3′ | *vca0867* promoter region amplification--contains *Spe*I restriction site tail |
| GT743 | 5′ AAGGATCCGAAAATGGCGTAGCAGCGAGG 3′ | *vca0867* promoter region amplification—contains *Bam*HI restriction site tail |

# RESULTS

**Cloning of each of the three putative CytR-CRP controlled target genes into the pBBR*lux* reporter plasmid.** In order to measure regulation for the genes *vc1021* (LuxO), *vca0053* (PNP), and *vca0867* (OmpW), a portion of each gene that contained the entire promoter region and a portion of the coding sequence was cloned into a pBBR*lux* plasmid as described in Materials and Methods. Successful constructs would permit transcription of the luciferase operon (*luxCDABE*) *in vivo* under control of the promoter region of each target gene (Lenz et al, 2004). After unsuccessful efforts to clone each gene insertion into a pBBR*lux* vector in a parallel experiment, successful clones were constructed over the course of multiple experiments with troubleshooting to enhance productivity. For each successful cloning procedure, PCR amplified gene regions (~400 bp) and the pBBR*lux* vector were digested with the correct restriction enzymes and ligated. After electroporation into *E. coli* S17λpir cells, individual colonies were screened for correct insertion. ~25 colonies were screened for *luxO* insertion, ~100 for *vca0867* insertion, and ~*125* for *vca0053* insertion. For each target gene, ~2 colonies exhibited the correct insertion and were preserved as -80°C stock cultures for future mating experiments. Example AGE results for creation of a construct can be found in Supplemental Figures S1-4. Sequencing results verified correct gene insertion into the pBBR*lux* plasmid with no mutations in the insert, thus the three plasmids were saved, namely pBBR*lux*-*luxO*, pBBR*lux*-*vca0867*, and pBBR*lux*-*vca0053.* An example sequencing result can be found in Figure S5.

**Mutants were constructed from isogenic strains.** To test the effects of CytR and CRP on regulation of the *lux*-based gene fusions constructed (as described in Materials and Methods), several mutant background strains were used in bioluminescence assays including WT, *ΔcytR*, and *Δcrp* (all containing or lacking the *tfoX** allele). However, to test for a potential QS role in regulation, *ΔhapR* (cells locked at LCD) and *ΔluxO* (cells locked at HCD) were also used as comparative background strains for the reporter plasmid. Initial bioluminescence assays for *luxO* (results described below), in particular, exhibited a need to mate the construct into additional background strains for proper comparison. Based on our current understanding of LuxO regulation in *V. cholerae* (Figures 1 and 2), a luxO D47E (strain that constitutively mimics LuxO~P (Freeman & Bassler, 1999)), *ΔluxO ΔcytR*, *ΔluxO Δcrp, Δqrr1-4,* and *Δcrp Δqrr1-4* (all +/- *tfoX**) mutants were considered as useful to determine if, and to what degree, CRP and CytR regulate gene expression in strains where known feedback loops have been inactivated. Additional matings with pBBR*lux-luxO* were performed with a variety of strains including: *luxO D47E* (+/- *tfoX**), *ΔluxO ΔcytR* (+/- *tfoX**), and *Δqrr1-4,* all of which were previously constructed (Table 1).

Failed attempts to delete CRP from the chromosome via allelic exchange using the pKAS vector resulted in utilizing an alternative method, specifically natural competence (as describe in Materials and Methods), for constructing a *ΔluxO Δcrp* and a *ΔluxO Δcrp tfoX** mutant. Colony PCR confirmed replace of the *crp* gene with the *kanR* gene in the two strains (PCR results not shown). The pKAS allelic exchange system (described in Materials and Methods) was used to

insert *tfoX** onto the chromosome of the *V. cholerae Δqrr1-4* mutant. The *Δqrr1-4 tfoX** quintuple mutant was also successfully made by this method (PCR results not shown). Insertion of *tfoX** onto the chromosome of the isogenic *Δcrp Δqrr1-4* mutant utilizing the pKAS allelic exchange system was unsuccessful and alternative methods were considered but not attempted. Deletion of *cytR* from the isogenic Δ*crp*::KanR and Δ*crp*::KanR *tfoX** strains via the pKAS allelic exchange system was confirmed via colony PCR (results shown in Figure S6).

## BIOLUMINESCENCE ASSAYS

**LuxO regulation by CRP and CytR may require alternative methods for detection.** To test whether *luxO* is not only CRP activated, but also CytR anti-activated, pBBR*lux-luxO* was introduced by conjugation into WT ($CytR^+$, $CRP^+$), Δ*luxO* ($CytR^+$, $CRP^+$), Δ*cytR* ($CytR^-$, $CRP^+$), Δ*crp* ($CytR^+$, $CRP^-$), and Δ*cytR* Δ*crp* ($CytR^-$, $CRP^-$) *V. cholerae* strains that carried the native *tfoX* allele (*tfoX*$^-$) or that constitutively expressed *tfoX* (*tfoX**). Initial lux readings exhibited decreased expression in the Δ*luxO tfoX** strain (data not shown), indicating that CRP regulation may be more detectable in mutants that lacked other known regulators of *luxO* (Figure 1). As described in the Introduction, the *qrr1-4* sRNAs repress *luxO*, therefore, pBBR*lux-luxO* was also introduced into a *V. cholerae Δqrr1-4* (lacks *qrr1-4* repression, but retains LuxO autorepression) strain and a Δ*luxO* (lacks *qrr1-4* repression and LuxO autorepression) strain by conjugation. Expression was also remeasured in a *luxO D47E* mutant (LuxO~P) to determine if phosphorylation state may affect potential CRP activation, and/or CytR anti-

activation. If CRP were to activate *luxO*, expression in a Δ*crp* mutant would decrease. If CytR were to exhibit anti-activation on *luxO* transcription, expression would increase in a Δ*cytR* mutant and decrease in strains that constitutively express *tfoX* (*tfoX**) relative to their isogenic counterparts, which carry the native *tfoX* allele (*tfoX*$^-$). If known regulators of *luxO* (Figures 1 and 2) compete with CRP activation, a more profound difference in *luxO-lux* expression would be expected in the +/- TfoX isogenic strains that lack the feedback loops for *luxO* regulation (Δ*luxO* and Δ*qrr1-4*) compared to that of WT.

The expression of *luxO* was measured in three separate experiments using three replicates of each strain. The mean of each triplicate measurement exhibited high standard error and results were not always reproducible. Three of the readings (two shown in Figures 11A and 11B) exhibited greater *luxO* expression in a CRP mutant (<10-fold), suggesting that CRP may have a negative effect on *luxO* expression, though alternative methods must be used to verify that observation. Expression of *luxO-lux* in a Δ*cytR* mutant, compared to isogenic WT, as well as *tfoX** mutants compared to their *tfoX*$^-$ counterparts also varied across different measurements (Figure 11). Specifically, for the measurements in Figure 11A, the Δ*crp* mutant carrying the native *tfoX* allele (*tfoX*$^-$*)* was statistically different than the isogenic WT strain (p-value <0.05) and a Δ*qrr1-4* mutant (*tfoX*$^-$*)* was also statistically different than the isogenic WT (p-value <0.005). All *tfoX** mutants were not statistically different than their isogenic counterpart (*tfoX*$^-$). For the measurements in Figure 11B, the Δ*luxO,* Δ*cytR,* and Δ*crp* strains (all *tfoX*$^-$) were statistically different than the isogenic WT strain (p-

value <0.05), while a Δ*cytR* Δ*cytR* mutant was also statistically different than the isogenic WT strain (p-value <0.005). The Δ*cytR tfoX**, Δ*luxO* Δ*cytR tfoX**, and *luxO* D47E *tfoX** strains were statistically different than their *tfoX*$^{-}$ counterparts. Figure 11C exhibits that strains containing the native *tfoX* allele (*tfoX*$^{-}$) are not statistically different than the isogenic WT strain. Only the Δ*cytR tfoX** mutant was statistically different than the *tfoX*$^{-}$ counterpart.

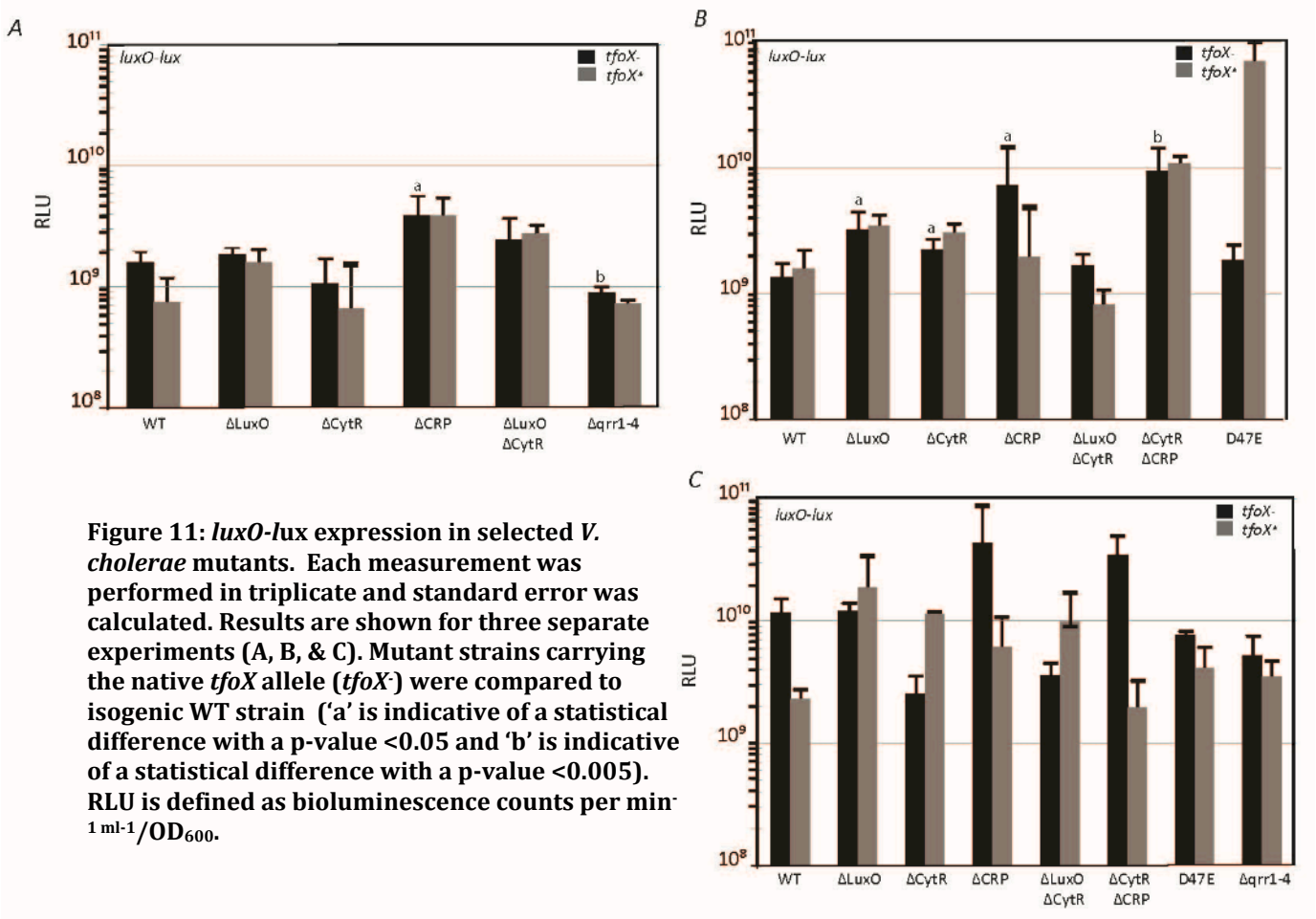


**Figure 11: *luxO-lux* expression in selected *V. cholerae* mutants. Each measurement was performed in triplicate and standard error was calculated. Results are shown for three separate experiments (A, B, & C). Mutant strains carrying the native *tfoX* allele (*tfoX*$^{-}$) were compared to isogenic WT strain ('a' is indicative of a statistical difference with a p-value <0.05 and 'b' is indicative of a statistical difference with a p-value <0.005). RLU is defined as bioluminescence counts per min$^{-1}$ ml$^{-1}$/OD$_{600}$.**

**Expression of *vca0053* (PNP) is activated by CRP, but not anti-activated by CytR, in *V. cholerae*.** Microarray analysis of a Δ*crp* mutant in *V.*

*cholerae* exhibited ~2.3 fold decrease in *vca0053* expression (Liang et al, 2007). To confirm this results and to test whether this gene is also CytR anti-activated, and perhaps regulated by QS, pBBR*lux-vca0053* was introduced by conjugation into WT ($QS^+$, $CytR^+$, $CRP^+$), Δ*luxO* ($QS^+$, $CytR^+$, $CRP^+$), Δ*hapR* ($QS^-$, $CytR^+$, $CRP^+$), Δ*cytR* ($QS^+$, $CytR^-$, $CRP^+$), Δ*crp* ($QS^+$, $CytR^+$, $CRP^-$), and Δ*cytR* Δ*crp* ($QS^+$, $CytR^-$, $CRP^-$) *V. cholerae* strains that carried the native *tfoX* allele (*tfoX*$^-$) or that constitutively expressed *tfoX* (tfoX*). If CytR indeed were to anti-activate *vca0053*, expression of *vca0053-lux* would be expected to increase in a Δ*cytR* mutant and decrease in strains that constitutively express *tfoX* (*tfoX**), relative to their isogenic counterparts, which carry the native *tfoX* allele (*tfoX*$^-$). WT (*tfoX*$^-$) The WT *V. cholerae* strain showed maximal expression of *vca0053-lux.* The Δ*hapR* and Δ*cytR* mutant strains (both *tfoX*$^-$) were statistically different than WT (p-value <0.05). The Δ*luxO,* Δ*crp*, and Δ*cytR* Δ*crp* (all *tfoX*$^-$) mutant strains were also statistically different than the isogenic WT strain (p-value <0.005). Specifically, the Δ*crp* and Δ*cytR* Δ*crp* mutants exhibited a <10-fold decrease in expression of *vca0053-lux.* Except for the Δ*cytR* Δ*crp* mutant, all *tfoX** strains were statistically different than their *tfoX*$^-$ counterparts. Measurements of *vca0053-lux* were repeated on three occasions and results are consistent. Figure 12 is one representative example of the three independent experiments measuring *vca0053-lux* expression.

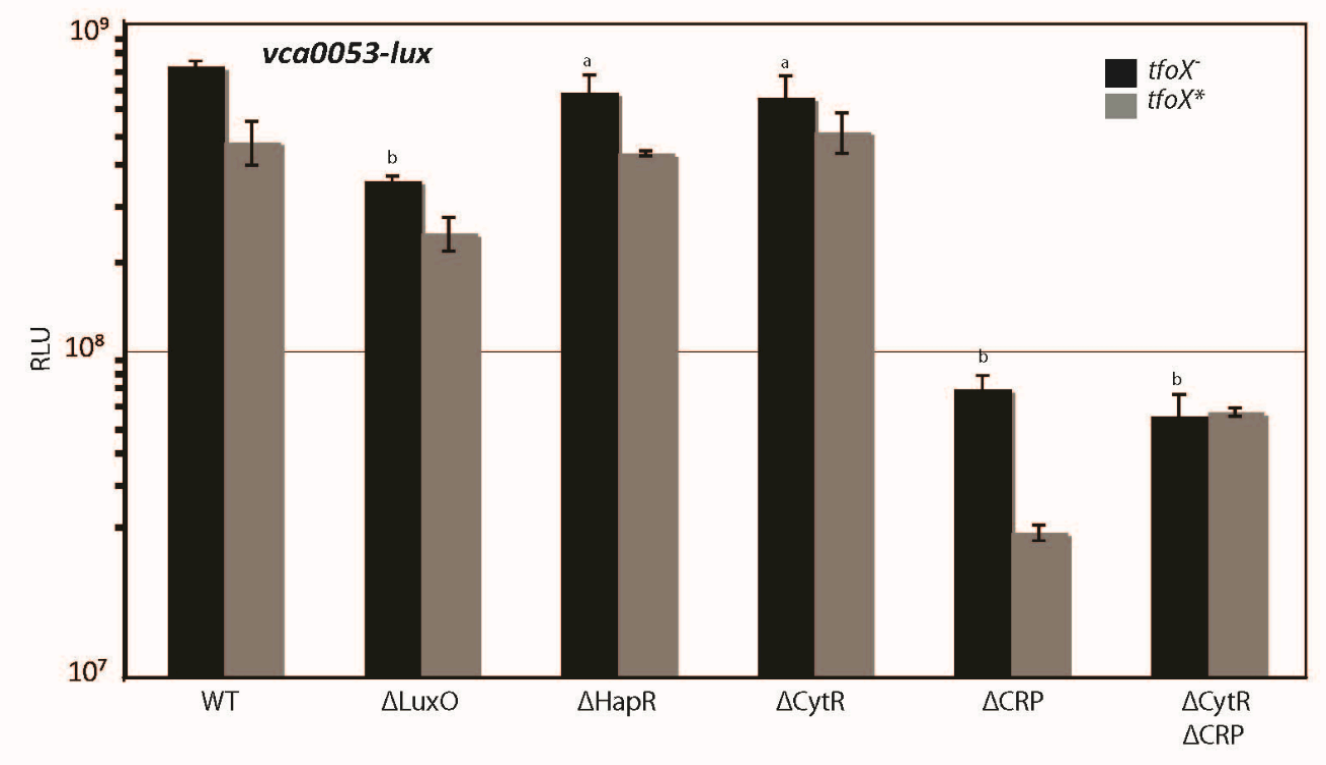


**Figure 12: Representative example of *vca0053-lux* expression in select *V. cholerae* mutant strains. Each measurement was performed in triplicate and standard error was calculated and shown by error bars. 'a' signifies a p-value <0.05 and 'b' signifies a p-value <0.005. Each TfoX- strain is statistical different than the TfoX* counterpart, except for Δ*cytR* Δ*crp*. RLU is defined as counts per $min^{-1}$ $ml^{-1}$/$OD_{600}$.**

**Expression of *vca0867* (OmpW) is activated by CRP, but not repressed by CytR, in *V. cholerae***. Microarray analysis of a Δ*crp* mutant in *V. cholerae* exhibited a ~2.5 fold decrease in *ompW* expression (Liang et al, 2007). To confirm this results and to test whether this gene is also CytR anti-activated, and perhaps regulated by QS, pBBR*lux-vca0867* was introduced by conjugation into WT ($QS^+$, $CytR^+$, $CRP^+$), Δ*luxO* ($QS^+$, $CytR^+$, $CRP^+$), Δ*hapR* ($QS^-$, $CytR^+$, $CRP^+$), Δ*cytR* ($QS^+$, $CytR^-$, $CRP^+$), Δ*crp* ($QS^+$, $CytR^+$, $CRP^-$), and Δ*cytR* Δ*crp* ($QS^+$, $CytR^-$, $CRP^-$) *V. cholerae* strains that carried the native *tfoX* allele (*tfoX*$^-$) or that constitutively expressed *tfoX* (*tfoX**). If CytR indeed were to exhibit anti-

activation on *ompW*, expression of *vca0867-lux* would be expected to increase in a Δ*cytR* mutant and decrease in strains that constitutively express *tfoX* (*tfoX**), relative to their isogenic counterparts, which carry the native *tfoX* allele (*tfoX*⁻). WT *V. cholerae* showed maximal expression of *vca0867-lux*. All mutant strains (*tfoX*⁻), Δ*luxO,* Δ*cytR,* Δ*crp,* Δ*cytR* Δ*crp* were statistically different than the isogenic WT strain. The Δ*cytR,* Δ*crp,* Δ*cytR* Δ*crp* mutants each exhibited a ~2.5 decrease in expression. Induction of *tfoX* (*tfoX**) had no significant effect compared to their isogenic counterparts. The Δ*hapR* strain carrying the native *tfoX* allele (*tfoX*⁻) was not measured because an overnight culture of the strain failed to grow, however, the Δ*hapR tfoX** strain exhibited a ~2.5 fold decrease in expression, suggesting that QS may play a role in the expression of this gene, as HapR may contribute to its expression. Figure 13 shows *vca0867-lux* expression in described strains. These measurements must be repeated to verify results.

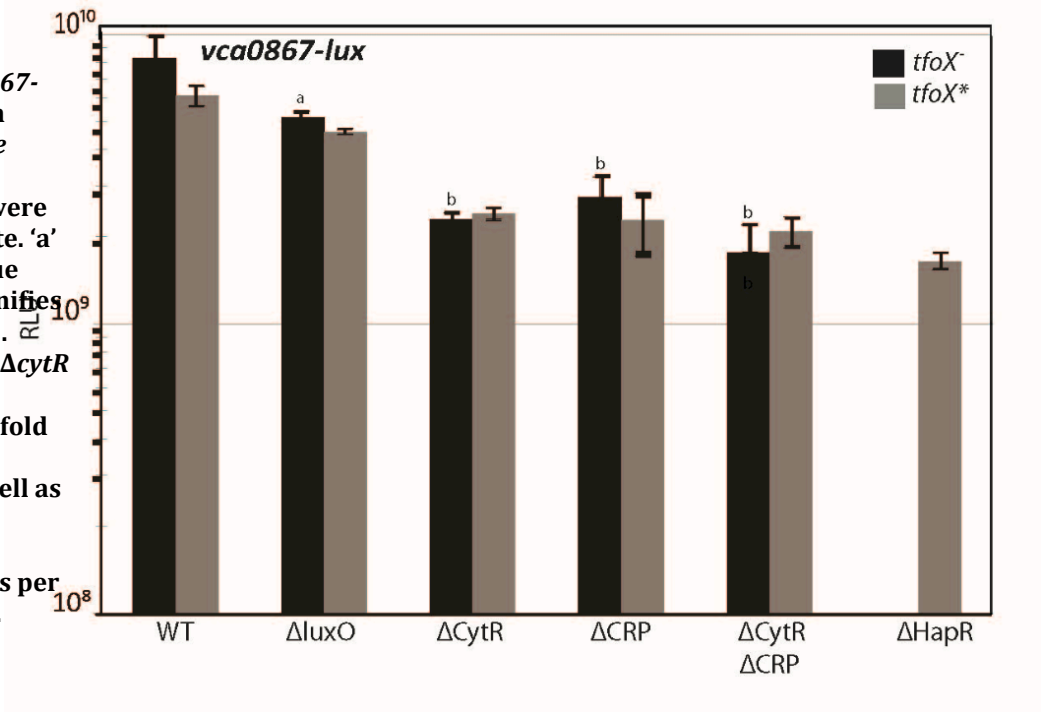


**Figure 13: *vca0867-lux* expression in select *V. cholerae* strains. Measurements were taken in triplicate. 'a' signifies a P-value <0.05 and 'b' signifies a P-value <0.005. Δ*crp*, Δ*cytR,* and Δ*cytR* Δ*crp* mutants exhibited a ~2.5 fold decrease in expression, as well as a Δ*hapR* (*tfoX**) mutant. RLU is defined as counts per $min^{-1}$ $ml^{-1}$/$OD_{600}$).**

# DISCUSSION

**Utilizing bioluminescence to monitor gene expression and its limitations in monitoring *luxO* expression.** Utilization of bioluminescence assays for characterizing gene regulation was used to indentify the genetic components of the QS pathway (Figure 1) and since then has become a useful tool to regulate gene expression (Lenz et al, 2004; Miller et al, 2002; van der Meer & Belkin, 2010; Yagur-Kroll & Belkin, 2011; Zhu et al, 2002). The *lux* readout for a reporter plasmid in a bacterial culture is dependent on numerous conditions. First and foremost, the bioluminescence operon, *luxCDABE,* codes for genes involved in an enzymatic reaction to produce light. Specifically, *luxAB* codes for a luciferase dimer that catalyzes the oxidation of a luciferin, which consists of a long-chain fatty aldehyde and a reduced flavin mononucleotide ($FMNH_2$). The *luxCDE* genes encodes proteins necessary for the synthesis of the aldehyde. This reaction requires molecular oxygen (Meighen, 1991). Therefore, when *luxCDABE* is transcribed under the regulation of the inserted promoter region of the target gene, the cells become bioluminescent. The amount of light produced can then be measured and quantified for comparison across at least two bacterial strain backgrounds. The methods for this study involved utilizing a liquid scintillation counter (described in Materials and Methods) to detect and measure the amount of light produced. Each measurement was an average of the amount of light produced by a culture of bacteria over a period of 30 seconds, measured in counts per $min^{-1}$. While measurements may vary on a daily basis due to the fact that light production is an enzymatic reaction, the relative

expression across compared strains typically stays the same, confirming that *lux* readings are a valuable and useful way to compare gene expression. However, because of this variation, it is difficult to compare expression in strains with different backgrounds when the difference in values is <3-fold (Hammer & Bassler, 2009). As shown in the results for *luxO* expression (Figure 11), inconsistency over several measurements indicate that any regulation of *luxO* by CRP and CytR may be below the level of detection for bioluminescence assays. Proposed modifications and alternative methods to monitor *luxO* expression in a Δ*crp* mutant and a Δ*cytR* mutant in efforts to determine their potential roles in *luxO* regulation are discussed in detail below.

**The role of CRP in the regulation of *vca0867* and *vca0053* and the contribution to understanding of CRP-CytR regulation of genes in *V. cholera.*** The gene *vca0053* (predicted to encode a nucleotide protein, PNP) was an interesting candidate to test for involvement in a DNA uptake pathway due to its potential role in nucleotide metabolism (Bzowska et al, 2000). Relative to WT *V. cholerae*, a Δ*crp* mutant exhibited a decrease in expression of *vca0053-lux* (Figure 12). These results are consistent with studies from the Benitez group identified in *V. cholerae* by microarray genes under control of catabolite repression (Liang et al, 2007). The bioinformatics analysis described in the Introduction also predicted potential two CRP binding sites of the *vca0053* promoter region. The reduction in *vca0053* transcription in the Δ*crp* mutant relative to the WT strain was significant and reproducible (Figure 12), supporting a model that CRP directly binds to the promoter region of *vca0053*. CytR does

not appear to play a highly detectable role in anti-activation of *vca0053*, as a Δ*cytR* mutant does not exhibit increased expression (Figure 12). Therefore, *vca0053* may be CRP activated; however it is not CytR anti-activated, and is an unlikely directly involved in the natural competence pathway described in Figure 4. Indeed the predicted CRP-2 site compared to the CRP-1 binding site appears to more closely match the consensus suggesting that a single CRP dimmer binds CRP-2 and not CRP-1. In future direction, possible methods for testing direct binding of CRP to the CRP-2 are discussed.

In a Δ*crp* mutant, *vca0867-lux* showed a ~2.5-fold decrease in expression relative to WT *V. cholerae* (Figure 13), suggesting that CRP may be required for gene activation. These results are also consistent with microarray studies from the Benitez group (Liang et al, 2007). However, microarray analysis does not address whether or not CRP directly acts on *ompW*, therefore, one interpretation of these observations it that like *vca0053*, CRP activates transcription. As with *vca0053*, bioinformatics predictions support direct biding of CRP to the *ompW* promoter. Methods to determine direct binding of CRP can be utilized and are described below. CytR does not play a detectable role in repression of *ompW*, as a Δ*cytR* mutant does not exhibit increased expression (Figure 13). Therefore, *ompW* appears to be CRP activated; however it is not CytR anti-activated and is also an unlikely candidate for significant involvement in our natural competence pathway described in Figure 4.

Regulation of genes by CRP and the CRP-CytR complex is not well characterized in *V. cholerae*. The model system for CRP activation and CytR

anti-activation described in the Introduction was characterized in *E. coli*. In *E. coli* CRP activates over 100 genes involved in metabolism. Of those ~100 genes, only 7 have been identified as being CytR anti-activated (Busby & Ebright, 1999; Valentin-Hansen et al, 1996). In *V. cholerae* only one gene, *udp* (uridine phosphorylase), has been shown to be directly regulated by CytR (Haugo & Watnick, 2002; Zolotukhina et al, 2003), suggesting that CytR in *V. cholerae* regulates genes quite different from those in *E. coli*. Uncovering the mechanism of a set of CytR regulation in *V. cholerae*, and therefore determining gene(s) involved in the natural competence pathway and nucleotide metabolism (Figure 4), is an area of interest for future studies by the Hammer lab. Proposed methods for future characterization of CRP activation, and CytR anti-activation, of *V. cholerae* genes are described below.

## FUTURE DIRECTIONS

**Alternative methods for detection of *luxO* expression.** LuxO has many characterized feedback loops for transcription, translation, and phosphorylation state (Figures 1 and 2) due to its critical role in the QS pathway (Ng & Bassler, 2009; Svenningsen et al, 2009; Tu et al, 2010). Each of these mechanisms for regulation varies depending on the growth state of the bacterial culture (Svenningsen et al, 2008). Lux readings, as described in Materials and Methods, were obtained with cultures that typically have reached HCD. Variations in cell density are corrected for in the equation for Relative Light Units (RLUs) by dividing the calculated light produced by the culture $OD_{600}$ (counts per $min^{-1}$ $ml^{-}$

$^{1}/OD_{600}$). However, extreme variations in culture cell density may have contributed to the dramatic affect on calculated light production specifically for *luxO* expression (Figure 11). Typically, the role of QS on gene regulation can be accounted for by measuring gene expression in strains locked at HCD (Δ*luxO*) or locked at LCD (Δ*hapR*), which eliminates the need to monitor growth phase during *lux* readings for many target genes. However, since *luxO* is involved in the QS pathway, and because it is highly regulated, more careful monitoring of cell growth phase may be necessary when comparing gene expression. This is particularly true when comparing a Δ*crp* mutant to the isogenic WT. In *E. coli*, CRP regulates over 100 genes, and a *V. cholerae* Δ*crp* mutant shows diminished growth in media, such as LB, that contains more complex carbon sources than glucose (data not shown). Even though differences in $OD_{600}$ were accounted for in the RLU equation, a drastic change in actual growth phase (and the amount of time spent in that particular growth phase) could have accounted for high standard error per reading and numerous fluctuations in readings across different days.

Variations in bioluminescence assay protocols, or alternatives methods, must be considered for monitoring *luxO* expression. The most logical step to consider would be to monitor *lux* production in both a WT and Δ*crp* mutant in a time course experiment, which could be accomplished by back diluting and overnight culture to the same $OD_{600}$ (for example 0.001) and measuring both *lux* and $OD_{600}$ at various time points during the growth for each strain. A plot of RLU vs. $OD_{600}$ would exhibit *luxO* expression during growth and levels of expression

could easily be compared across a WT and Δ*crp* mutant. If this method provides confident results, the other constructed strains (mentioned in Materials and Methods and Table 1) for *luxO* regulation can be measured over a series of several hours (as described in (Miller et al, 2002)) to determine the level of CRP regulation compared to other known regulators, as well as if CytR plays a role in anti-activation. If CRP were indeed required for *luxO* expression, the entire readout for the *lux* time course in a Δ*crp* mutant would be expected to be lower than the isogenic WT. If *luxO* is anti-activated by CytR, expression would increase in a Δ*cytR* mutant.

Alternative methods for monitoring *luxO* expression could also be considered. Since *lux* measurements, as described in Materials and Methods, provide only an average for a culture, a method, such as flow cytmometry, could be used to monitor light produced by each individual cell. While an average light reading would also be calculated for a tested culture, graphical analysis of the results may exhibit more concise differences in gene expression (Giepmans et al, 2006). Levels of *luxO* mRNA in the cell could also be compared between a WT and Δ*crp* mutant, as well as other strains, by quantifying *luxO* RNA levels directly from cell lysates. A method for quantifying cellular RNA levels, via qRT-PCR, at various time points for *luxO* has been previously described (Tu et al, 2010). Again, this proposed method would require harvesting of cells at similar points in a growth curve (at various $OD_{600}$) to eliminate variations in *luxO* mRNA levels as a result of growth phase.

**Methods for verifying CRP activation and for identifying direct CRP-CytR interactions with genomic DNA.** To test whether predicted CRP-activated genes, such as *vca0867* (OmpW) and *vca0053* (PNP), directly interact with CRP at the predicted CRP binding sites *in vivo*, genetic manipulations could be performed to mutate the predicted CRP binding site on the reporter plasmid of the gene. Single or several nucleotides can be mutated on each *lux*-based reporter plasmid in the sequence predicted to bind CRP and *lux* measurements can be compared to a WT strain containing a reporter plasmid containing the native CRP binding site. To be considered as CRP activated, a *lux*-reporter plasmid with the promoter region of the gene (as described in Materials and Methods) must be introduced by conjugation into a WT strain. Lux levels must then be compared to a WT strain carrying a similar plasmid that contains mutations at the predicted binding site. If CRP directly binds to the target gene (and therefore to the reporter plasmid), as we predict for *vca0053* and *vca0867* CRP in a WT strain would not be able to bind to either reporter plasmid with a mutation in the predicted CRP binding site and transcription would not be activated. The lux reading in this example would be similar to those obtained in a Δ*crp* mutant.

One *in vitro* method for characterizing CRP binding to the promoter region of target genes is electrophoretic mobility shift assays (EMSAs). To perform an EMSA, the CRP protein must be purified and added to radiolabeled DNA from the promoter region target gene. All samples must be run on a gel for comparison. If the CRP protein binds to the CRP binding site on the DNA, a gel

shift would occur, resulting in a higher, slower running band compared to the radiolabeled DNA alone (Garner & Revzin, 1981). EMSAs can also be combined with DNase footprinting, in which DNA that is not bound to the protein is degraded by a nonspecific DNase enzyme, then protein-DNA complex is disrupted, and the remaining DNA sequence (that was once bound to CRP) is amplified and sequenced (Brenowitz et al, 1986).

Perhaps the most ambitious method for identifying genes in *V. cholerae* that are CRP activated would be to combine chromatin immunoprecipitation (ChIP) with microarray analysis in a relatively new method called ChIP-chip (for a minireview (Buck & Lieb, 2004)). This method provides a genome wide analysis of *in vivo* DNA binding sites for a given protein, such as CRP. Briefly, cells are grown and fixed with formaldehyde, which creates crosslinks between the DNA binding proteins and the DNA. The DNA is then sheared by sonication to create DNA fragments ~1kb or smaller and the resulting DNA fragments, with the protein of interest, are then selected for most notably through immunoprecipitation with a protein-specific antibody. The DNA-protein complex is broken and the DNA is purified and labeled with a probe and hybridized to a microarray. The specific DNA binding site for the desired protein, such as CRP, can be determined via computational methods (Buck & Lieb, 2004). A bioinformatics search can be performed on the genes known to directly interact with CRP in order to predict those that may be CytR anti-activated. This approach would significantly improve the chances of characterizing a gene that is

both CRP activated and CytR anti-activated, thus leading to the identification of *gene* X involved in the proposed natural competence pathway (Figure 4).

Characterization of CRP and CytR regulation is crucial to understand how *V. cholerae* becomes naturally competent to take up DNA in the presence of chitin and QS autoinducers. Since natural competence can promote HGT, uncovering additional regulatory mechanisms for QS and natural competence in *V. cholerae* through exploration and utilization of various molecular techniques may provide insight into the evolution of this pathogen. An understanding of how and when bacteria, such as *V. cholerae,* become competent to take up exogenous genes can provide a deeper appreciation for the molecular evolution of microbes, that may be utilized to combat potential pathogens and to promote the success of beneficial microorganisms.

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## SUPPLEMENTAL FIGURES

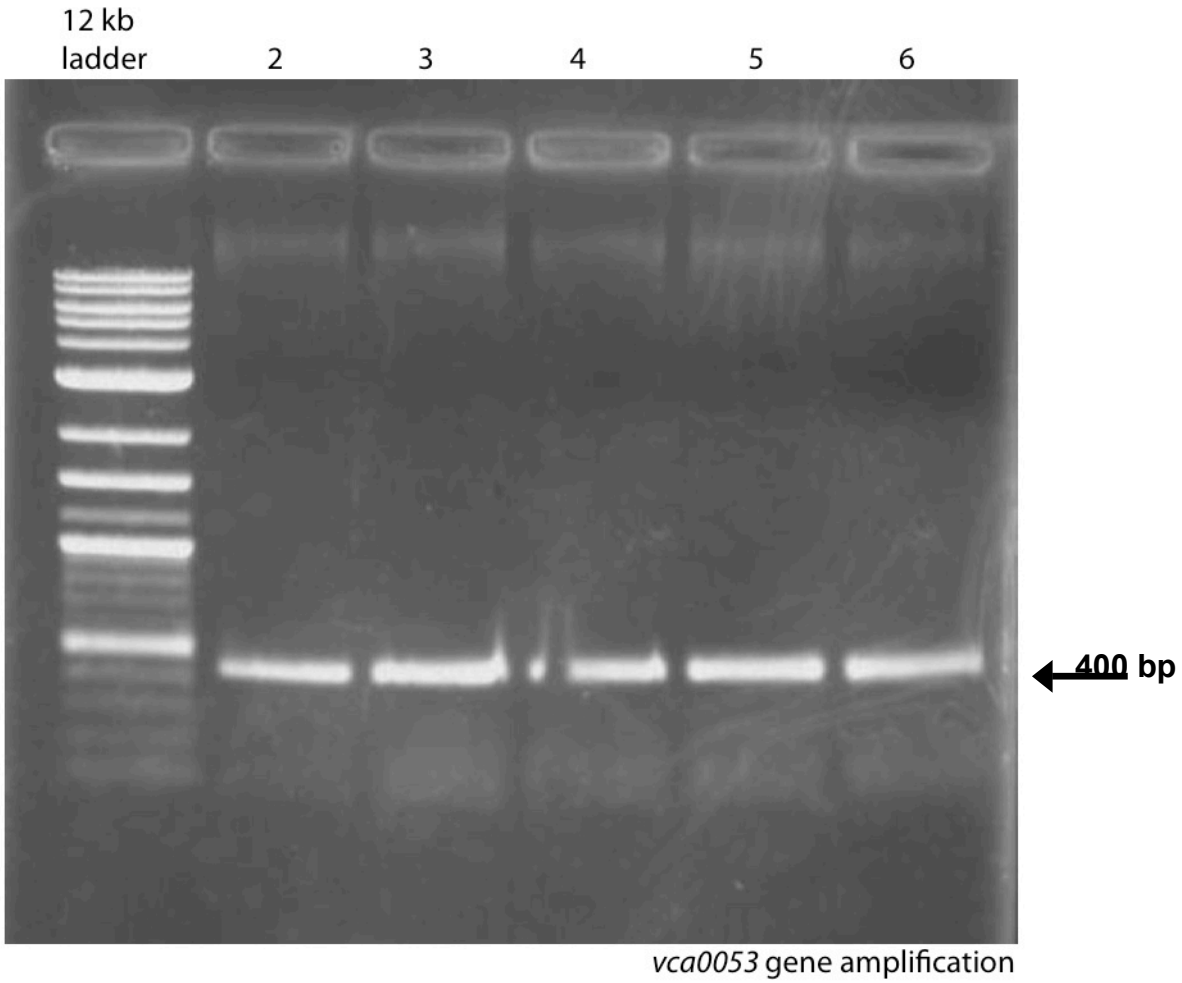


**Figure S1: PCR reaction for target gene fragment amplification. Lane 1 is a 12 kb ladder, lanes labeled 2-5 are amplified regions for the *vca0053* gene fragment.**

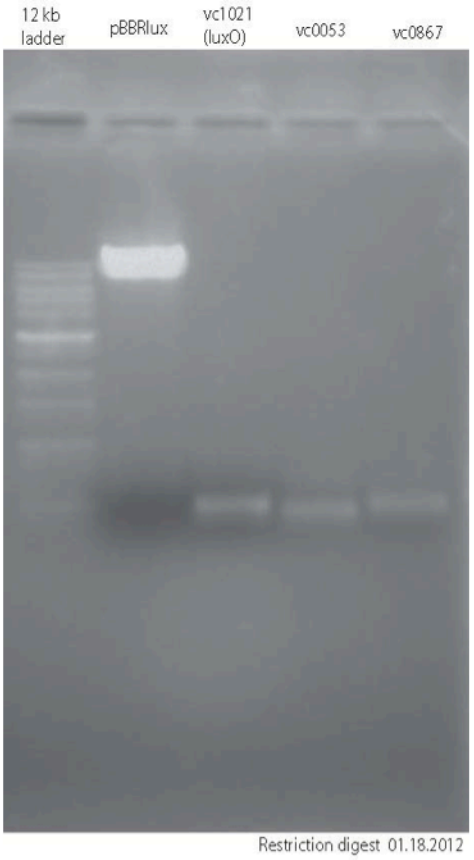


**Figure S2: Restriction digest gel purification. Lane 1 is a 12 kb ladder. Lane 2 is a digested pBBR*lux* plasmid, lanes 3-4 are digested gene fragments.**

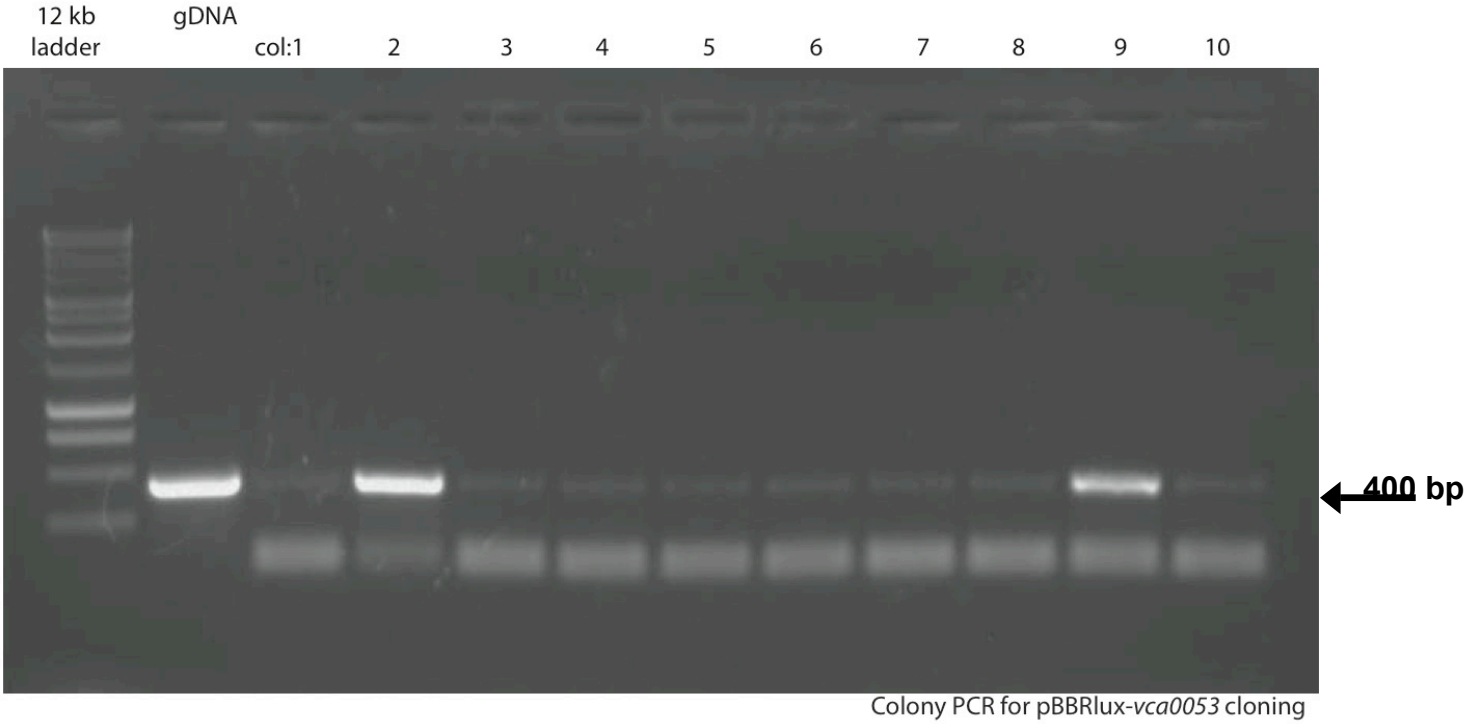


**Figure S3: Colony PCR to test for insert into pBBR*lux* plasmid. Lane 1 is a 12 kb ladder. Lane 2 is gDNA (positive control). Lanes 3-12 are colonies tested. In this example, colonies 2 and 9 were positive for insertion.**

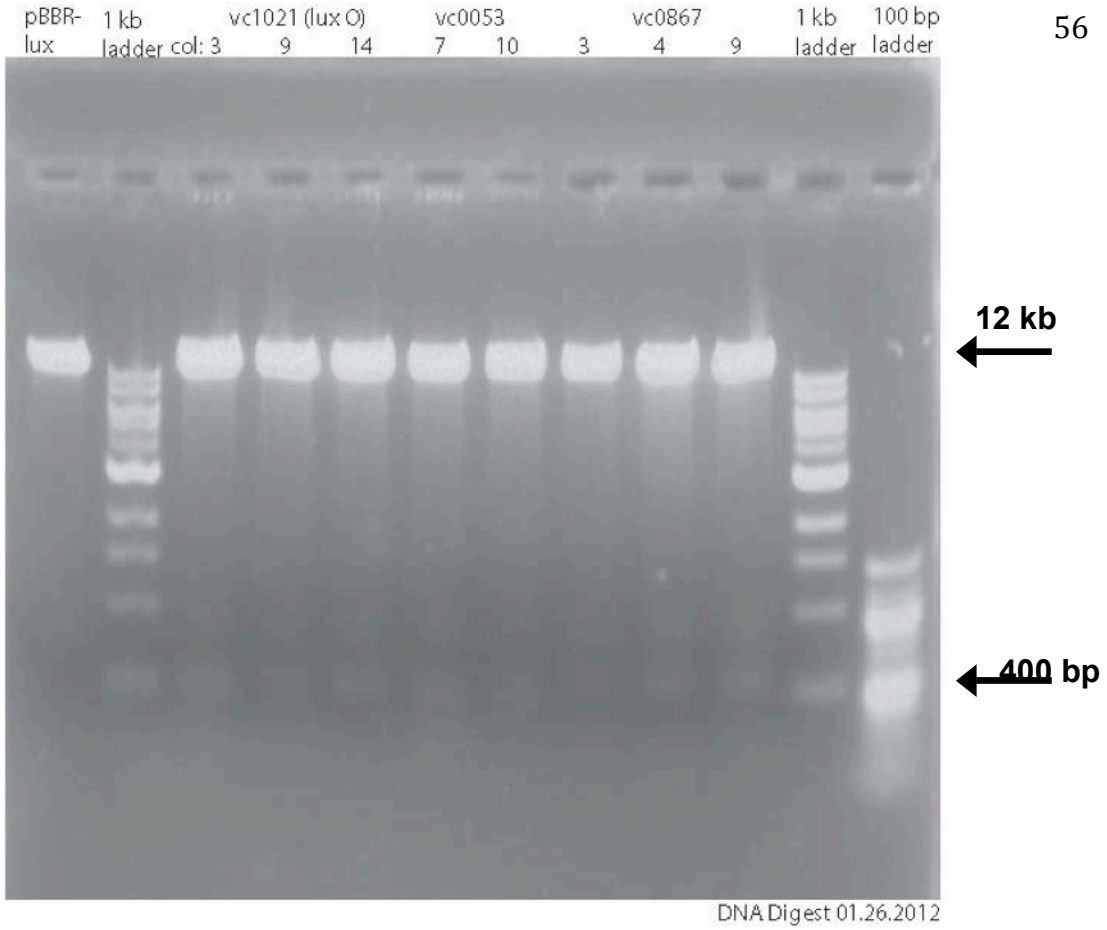


**Figure S4: DNA digest testing for gene insertion into the pBBR*lux* vector. Lane 1 is the vector control. Lanes 2 and 11 are 12 kb ladders. Lane 12 is a 100 bp ladder. Lanes 3-10 are tested colonies. All tested colonies have ~12 kb band for pBBR*lux* vector. Faint banding at ~400 bp mark is suggestive of insert.**

**Figure S5: Sequencing results example for *vca0053* insert. The top line is sequence results for gene insertion with primer GT741. The second line is the sequence for the *vca0053* fragment. The third line is sequence results for gene insertion with primer GT740, and the 4th line is a consensus sequence. Yellow is indicative of the correct sequence.**

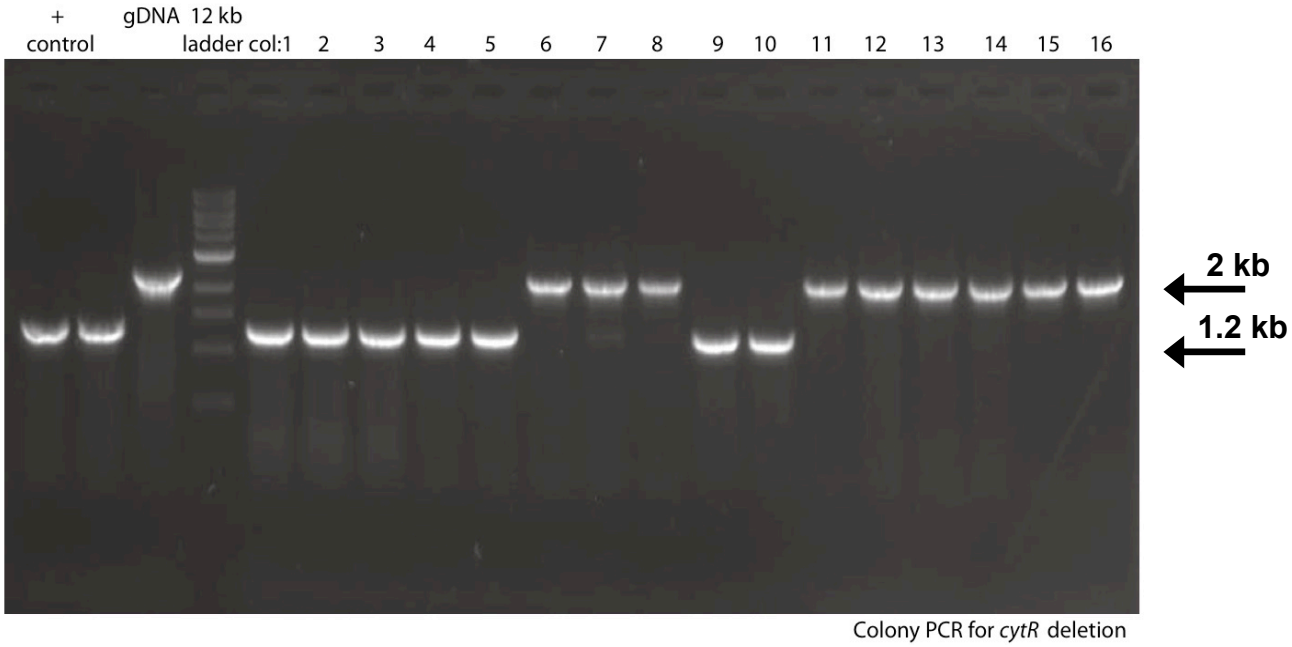


**Figure S6: Colony PCR for *cytR* deletion from *V. cholerae* chromosome. Lanes 1-2 are controls for proper deletion. Lane 3 is gDNA (contains WT *cytR*). Lane 3 is a12 kb ladder. Lanes 5-20 are tested colonies. In this example, colonies 1-5 and 9-10 appear to have the proper deletion.**