THE EFFECTS OF CARBON SOURCES ON CAMP-CRP-MEDIATION REGULATION IN *VIBRIO FISCHERI*

by

DEANNA MARIE COLTON

(Under the Direction of Eric V. Stabb)

ABSTRACT

The Proteobacterium *Vibrio fischeri* induces bioluminescence upon establishing its light-organ symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes.* This bioluminescence is controlled by pheromone-mediated signaling, which in turn is influenced by many environmental factors, one of which is a carbon source. In particular, glucose affects pheromone regulation, apparently through the activity of the cAMP-receptor protein CRP and the second messenger cAMP, which is generated by CyaA. In the absence of glucose, cAMP-CRP stimulates many catabolic operons in Proteobacteria, and *crp* is important in host colonization for other vibrios. Therefore, I became interested in cAMP-CRP's role in the light-organ environment, and how a carbon source translates into changes in cAMP-CRP activity. My first goal was to investigate cAMP-CRP's role in the symbiosis and during growth in culture. Both Δ*cyaA* and Δ*crp* mutants were attenuated in host colonization, and cAMP-CRP activity measured from a CRP-dependent transcriptional reporter was elevated in the symbiosis. In culture, glucose improved the growth of Δ*cyaA* and Δ*crp* mutants and decreased cAMP-CRP activity. *N*-acetylglucosamine (NAG)

similarly improved the growth of the mutants, although it did not decrease cAMP-CRP activity. My results suggest that CRP is active in *V. fischeri* during colonization and that glucose is not a major carbon source in the light organ. My second goal was to determine how cAMP and CRP levels are controlled in *V. fischeri*. In *Escherichia coli*, glucose lowers intracellular cAMP and CRP levels. However, despite lowered cAMP-CRP activity, I found higher intracellular cAMP levels in the presence of glucose. I showed that the proteins CyaA, CpdA, CpdP, and $EIIA^{Glc}$, along with an unknown transporter, control cAMP levels; however, neither cAMP concentration nor CRP levels seemed to explain the decrease in cAMP-CRP activity in glucose-grown cells. I hypothesize that CRP is being regulated by a post-translational mechanism, such as protein acetylation. This dissertation illustrates the importance of cAMP-CRP-mediated regulation in the *V. fischeri-E. scolopes* symbiosis and suggests there are yet unknown means by which glucose regulates cAMP-CRP activity in Proteobacteria.

INDEX WORDS: *Aliivibrio*, *Photobacterium*, CAP, dextrose

THE EFFECTS OF CARBON SOURCES ON CAMP-CRP-MEDIATION REGULATION IN *VIBRIO FISCHERI*

by

DEANNA MARIE COLTON

B.S., Biology, Virginia Tech, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2014

THE EFFECTS OF CARBON SOURCES ON CAMP-CRP-MEDIATION REGULATION IN *VIBRIO FISCHERI*

by

DEANNA MARIE COLTON

Major Professor: Eric V. Stabb

Committee: Timothy R. Hoover
Ellen L. Neidle
Lawrence J. Shimkets

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
August 2014

DEDICATION

This dissertation is dedicated to my big brother, Adam. He is an inspiration for his creative and adventurous outlook on life. I have been grateful to be living vicariously through him all these years.

ACKNOWLEDGEMENTS

I am thankful for my wonderful classmates and friends that have gotten me through my graduate career and especially to my lab members that make the long days not so bad. My parents have been a source of motivation and inspiration to me through all their hard work. I would like to thank my Dad for giving me the 'science gene' and looking over this dissertation and my Mom for our daily chats on my drive home from lab. I would especially like to thank my husband, Aaron, for all his love, support, and encouragement throughout this process. I would also like to thank Eric Stabb for all his mentoring and the rest of my committee for their valuable advice.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

Bacteria use diffusible signaling molecules, or "pheromones," to regulate gene expression and to coordinate certain behaviors such as bioluminescence, biofilm formation, virulence, or motility. These group behaviors often contribute to successful bacteria-host interactions, whether they are mutualistic symbioses or pathogenic infections. Bacterial pheromones accumulate in a cell-density dependent manner and once a threshold concentration of the pheromone is reached, the signal binds a cognate receptor, which directly or indirectly activates transcription of genes needed to promote a certain behavior. Because of the cell-density dependence for pheromone accumulation, such signaling is often called "quorum sensing." However, bacteria also change their group behaviors in different environments and niches where they are faced with fluctuating factors such as changes in nutrient sources or the presence of other bacteria in the community (18, 41). These conditions can affect the accumulation of pheromone signals through constraints on pheromone diffusion, changes in signal degradation, or regulation of pheromone synthases (18). In this way, bacteria respond to changing environments in part by controlling certain group processes using pheromone-mediated regulatory circuits that require both a "quorum" of cells and an appropriate external environment.

In the remainder of this chapter, I will describe bacterial pheromone-mediated regulation by two structurally different types of pheromones, *N*-acyl-homoserine lactones (AHLs) and a signal

called AI-2. In addition, I will discuss the pheromone-controlled *lux* system underlying bioluminescence in *Vibrio fischeri*, the *Vibrio*-squid light-organ symbiosis, and evidence for cAMP-CRP-mediated regulation of bioluminescence in this system. I will then review similar signaling systems in other bacteria and the regulatory input of cAMP-CRP into these systems. Lastly, I will describe the importance of environmental factors in regulating bioluminescence and how specific carbon sources could act as environmental cues to control the cAMP-CRP regulon in *V. fischeri*.

Pheromone-mediated regulation

First discovered in *V. fischeri*, LuxR-LuxI-type AHL signaling systems are well conserved and have been reported in over seventy proteobacterial genera (18). The LuxI-type proteins synthesize AHLs (Fig. 1.1), which can have different acyl chain lengths or decorations

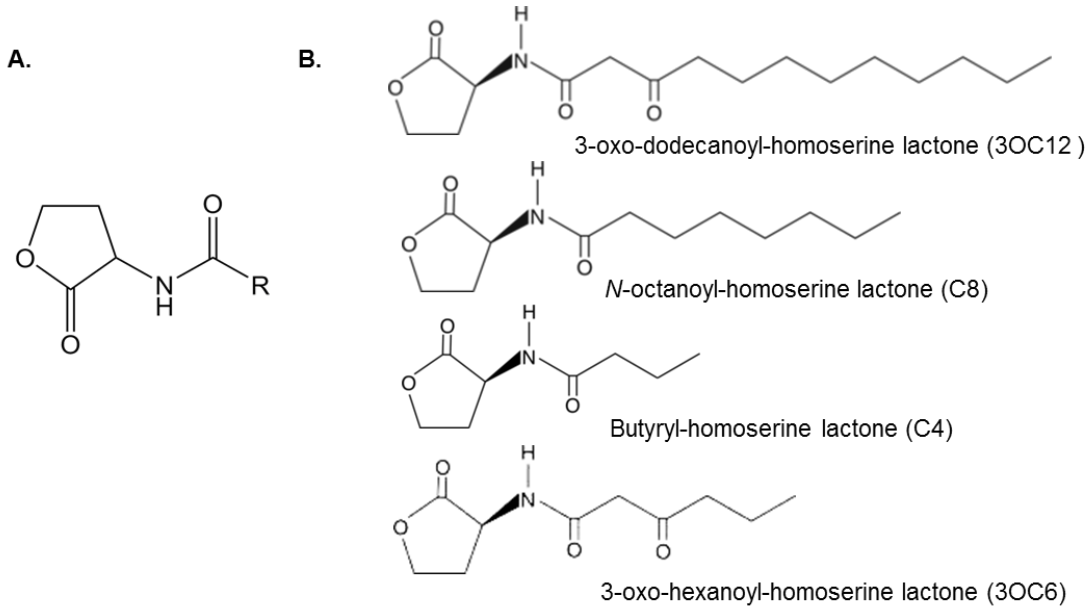

Figure 1.1: Structures of AHL pheromones produced by many Proteobacteria. **(A)** Structure of an AHL; "R" represents a variable acyl chain. **(B)** Examples of AHLs with different chain lengths and decorations such as an oxo group.

such as a hydroxyl group. AHLs are generated by the formation of an amide bond between *S*-adenosyl methionine (SAM) and an acyl moiety of the cognate acyl carrier protein (ACP) (18, 62, 105). The availability of the ACP substrates and the specificity of the synthases for different acyl-ACPs allow bacteria to produce different AHLs (Fig. 1.1B) (18). For example, YtbI of *Yersinia pseudotuberculosis* can synthesize up to 24 different AHLs (18, 112), whereas LuxI of *Vibrio fischeri* predominantly produces only one. The N-terminal portion of LuxI homologs contains six conserved residues found throughout the LuxI family that are important for AHL synthase activity (18, 63). The C-terminal region of LuxI homologs may also play an important role by providing specificity with respect to the acyl-ACP substrate (63).

LuxR and LuxR-type proteins are transcriptional regulators that bind to their cognate AHLs and subsequently bind to DNA to regulate the transcription of target genes as described below. LuxR homologs have conserved amino acids (162) and structural similarities in the N- and C-termini, corresponding to the sites of AHL interaction and DNA binding, respectively (18, 54, 146, 173). Interestingly, some bacteria in the *Vibrionaceae* family have AHL-based signaling systems that are structurally and functionally unlike the LuxR-LuxI system. One of these is the AinS/AinR system in *V. fischeri*, which will be discussed in greater detail below.

Another type of pheromone, collectively known as AI-2 (Fig. 1.2), is more universal, being produced by both Gram-positive and Gram-negative bacteria (31, 149, 166), suggesting a role for inter-species communication. AI-2 signaling in various bacteria controls bioluminescence, virulence, and biofilm formation (114, 152, 166). AI-2 was first identified as a regulator of luminescence in *Vibrio harveyi* and was shown to be made from SAM, which is a methyl donor for different cellular processes (8, 28). This use of SAM generates a toxic byproduct, *S*-adenosyl homocysteine (SAH), that is then recycled by the enzymatic activities of

Pfs and LuxS, the latter of which is known as the AI-2 synthase (8, 31). LuxS converts *S*-ribosil homocysteine (SRH) to homocysteine (Fig. 1.2) resulting in an unstable byproduct, 4,5-dihydroxy-2,3-pentanedione (DPD), which undergoes a non-enzymatic conversion to a *R* or *S* form of 2,4-dihydroxy-2-methyldihydro-3-furanone (DHMF). *R*-DHMF undergoes hydration to form the AI-2 molecule used by *E. coli* and *Salmonella* whereas the *S*-DHMF will also undergo hydration but then complexes with borate to form AI-2 recognized by *V. harveyi* (Fig 1.2) (152). Thus, related but structurally distinct compounds are collectively referred to as AI-2. *V. fischeri* utilizes both AI-2 and AHL-based signaling, which will be explained further below.

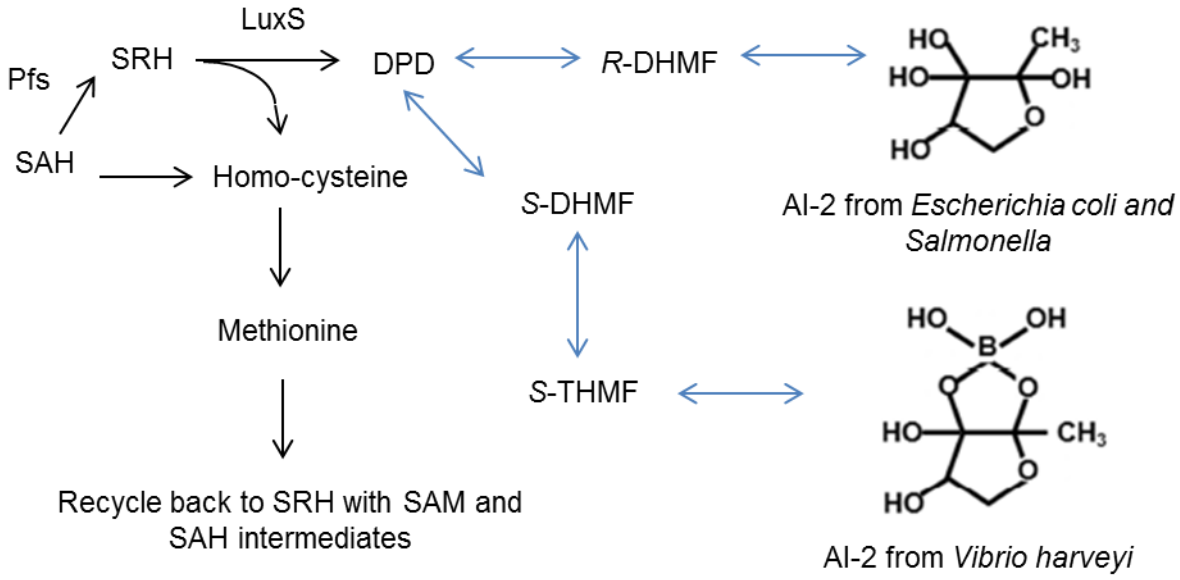

Figure 1.2: Synthesis of AI-2. Blue arrows represent the non-enzymatic reactions in which two isomers *R*- or *S*- form AI-2 for *E. coli* and *Salmonella* and AI-2 for *V. harveyi*, respectively. Abbreviations include: SAM, *S*-adenosyl methionine; SAH, *S*-adenosyl homocysteine; SRH, *S*-ribosil homocysteine; DPD, 4,5-dihydroxy-2,-3-pentanedione; DHMF, 2,4-dihydroxyl-2-methyldihydro-3-furanone; and *S*-THMF, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran.

The *V. fischeri –E. scolopes* symbiosis and the *lux* operon

The *V. fischeri-E. scolopes symbiotic infection*

One model strain for studying AHL- and AI-2-based signaling is *V. fischeri* ES114, which is an isolate from the light organ of the Hawaiian bobtail squid, *Euprymna scolopes* (12). This symbiotic relationship provides *V. fischeri* with nutrients, and *V. fischeri* populations are higher in environments occupied by the squid (81). In exchange, the squid utilizes the bacterial luminescence presumably as a camouflaging behavior called counterillumination to obscure the squid's silhouette from predators at night (70, 143, 156).

V. fischeri makes up less than 0.1% of the bacterial community in the environment where the host lives, and yet only *V. fischeri* colonizes the light organ of *E. scolopes* (96). After hatching, the juvenile squid are infected with *V. fischeri*, which triggers the development of the light organ where *V. fischeri* resides (99). Once inside the light organ, *V. fischeri* divides rapidly and induces its luminescence (96). Although the *luxICDABEG* (*lux*) genes that are responsible for luminescence are induced at different times in distinct light-organ microenvironments, most if not all symbiotic cells have induced luminescence within 24 hours post-infection (40). Luminescence is a colonization factor for *V. fischeri*, and pheromone signaling is required for its induction in the host (15, 153).

Luminescence and its regulation in V. fischeri

V. fischeri generates bioluminescence using proteins encoded by the *lux* operon. The enzyme luciferase, LuxAB, catalyzes the light-generating reaction, by converting a reduced flavin mononucleotide ($FMNH_2$), a long-chain aliphatic aldehyde (RCHO), and oxygen to oxidized FMN, aliphatic acid (RCOOH), water, and light (Fig. 1.3) (90). The aliphatic acid

reductase complex (LuxCDE) recycles the acid to aldehyde, and LuxG regenerates $FMNH_2$ (19, 45, 46, 90). In addition to the energy devoted to Lux protein synthesis, this process consumes reducing equivalents along with oxygen, potentially competing with energy-generating pathways for these substrates, and it hydrolyzes ATP in regenerating the aldehyde substrate. Perhaps not surprisingly, expression of luminescence can slow the growth of bacteria, and dim or dark mutants may outgrow wild-type in culture (15).

Because luminescence is an energetically expensive process in *V. fischeri*, the *lux* operon is tightly regulated. Notably, luminescence is controlled in a cell-density dependent manner and is not highly expressed in dilute planktonic cells. Such regulation is easily rationalized, because the luminescence output of a lone single cell would be too low to be detected by any biological system. The bacteria monitor their own population density through diffusible pheromones, which accumulate in the surrounding environment. At low cell densities, the pheromones are at low concentrations and unable to induce luminescence until they have reached a higher concentration that can only be achieved at higher cell densities (43, 55, 110). Once the threshold concentration of pheromone is reached, the *lux* operon is induced.

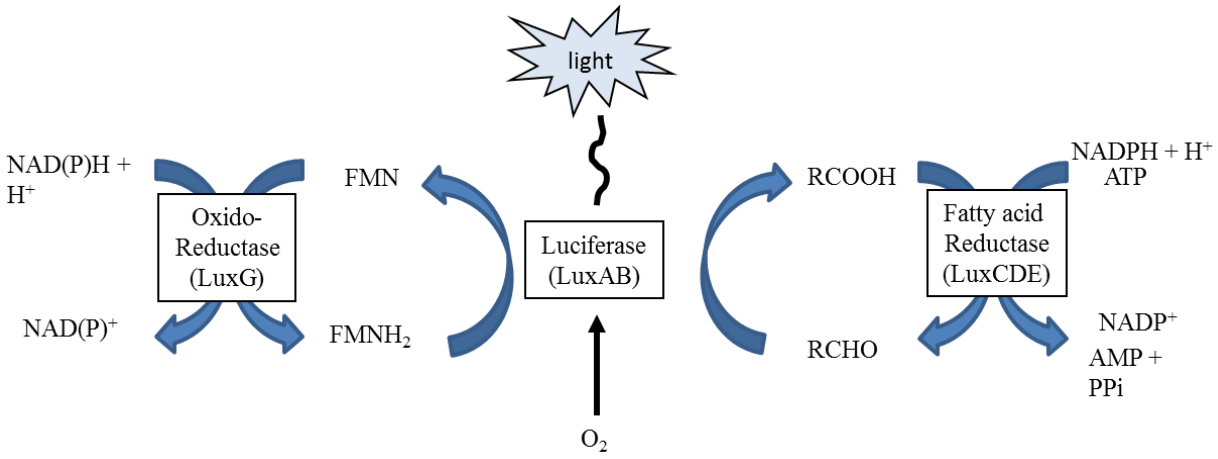

Figure 1.3: Light reaction involving LuxCDABEG proteins. LuxAB is luciferase and LuxG and LuxCDE are involved in recycling the products back into the substrates.

The *lux* operon also contains the pheromone synthase, LuxI, which produces 3-oxo-hexanoyl-homoserine lactone (3OC6) (Fig 1.1). Once 3OC6 accumulates, it binds and activates the *lux* transcriptional activator, LuxR (43, 44, 72, 137, 138). Activated LuxR can then bind the promoter of *luxICDABEG* at a *lux* box sequence to promote transcription, resulting in a positive feedback loop in which more LuxI and 3OC6 are synthesized to activate more LuxR (Fig. 1.4) (136). Pheromones like 3OC6 are often called "autoinducers", because of their self-stimulatory regulatory role.

As mentioned above, *V. fischeri* has an additional AHL synthase, AinS, which

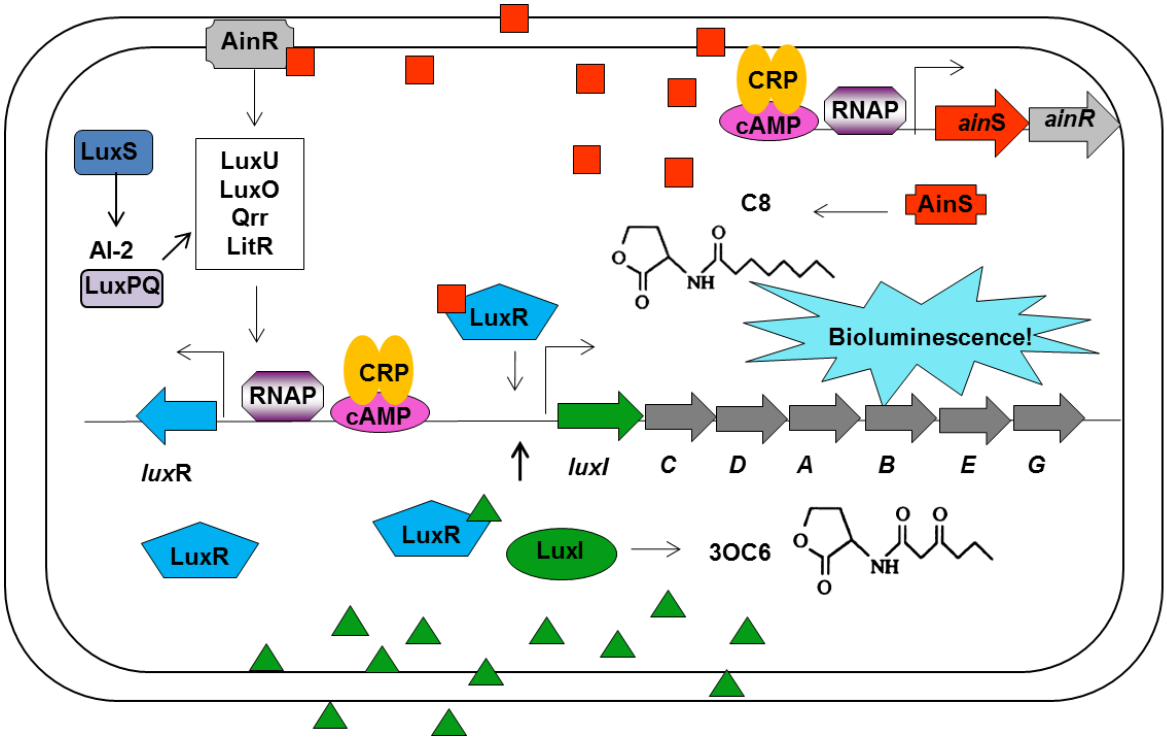

Figure 1.4: Pheromone-mediated regulation of *lux* operon in *V. fischeri*. LuxI and AinS produce AHL signals 3OC6 and C8, respectively, which combine with LuxR to stimulate transcription of *luxICDABEG*. LuxS produces AI-2. C8 or AI-2 along with their cognative receptors, AinR or LuxPQ, respectively, stimulate LuxR expression through a circuit involving LuxU, LuxO, Qrr, and LitR. In the presence of C8 or AI-2, this regulatory cascade results in transcriptional activation of LuxR by LitR. cAMP-CRP binds to both the *ainSR* and *luxR* promoters and activates their transcription (91).

synthesizes *N*-octanoyl-homoserine lactone (C8) (Fig. 1.1) (57, 79, 80). Although a weaker inducer than 3OC6, C8 also binds and activates LuxR (Fig. 1.4). Lupp *et al.* demonstrated that maximal induction of the *lux* operon requires both AHLs; however, C8 autoinduction precedes that of 3OC6 (89). It is believed that at moderate cell density, C8 autoinduction is prominent; and that at the higher cell density found during an established symbiosis, 3OC6 autoinduction is most important (90).

C8 also stimulates LuxR expression through a circuit involving LuxU, LuxO, Qrr, and LitR (Fig. 1.4) (142). AI-2 signals through this pathway as well, with both C8 and AI-2 signaling converging at LuxU. Based on similar systems in other *Vibrio* species (87, 97, 102), binding of C8 or AI-2 to their cognate receptors, AinR or LuxPQ, respectively, change the relative kinase and phosphatase activities toward LuxU (52, 151). Whereas in the presence of C8 or AI-2, kinase activity (but not phosphatase activity) of AinR and LuxPQ decrease leading to less phosphorylated LuxU and therefore less phosphorylated LuxO (51, 87). On the other hand, in the absence of C8 and AI-2, the cognate receptors have increased phosphatase activity to phosphorylate LuxU which then phosphorylates LuxO, a negative regulator of luminescence (104). Phosphorylated LuxO together with sigma 54 activates transcription of a gene encoding a small RNA (*qrr*) (82, 104) that in combination with RNA chaperone Hfq, leads to degradation of target mRNAs, notably *V. fischeri*'s *litR* transcript. Therefore, the presence of C8 or AI-2 contribute to the positive regulation of LuxR by LitR, a transcriptional activator that binds to the *luxR* promoter (49, 88), thus providing a link between these pheromones and the priming of the LuxR-LuxI system. However, AI-2-mediated regulation of luminescence appears minor under the conditions tested to date (87), whereas *ainS* and *ainR* play a larger role.

Environmental regulation of luminescence

Although high cell density is required for pheromone signaling and bioluminescence, it is not the whole story. Regulators of luminescence that have been discovered include ArcA, the response regulator of the redox-responsive ArcA/ArcB two-component regulatory system, which binds upstream of *luxI* and represses luminescence in culture (14). It was also shown that Mg^{2+} influences luminescence by both PhoQ-dependent and -independent mechanisms (92). Several other genes have likewise been shown to control luminescence (92). Among the first regulators of luminescence discovered was cAMP-CRP, which is discussed below and is believed to connect a carbon source, glucose in particular, with regulation of luminescence through the modulation of *luxR* and *ainS* (Fig. 1.4).

CRP and the second messenger cAMP

cAMP-CRP

The cAMP receptor protein (CRP) combines with cAMP to form a regulatory complex that binds DNA, thereby promoting transcription of genes encoding catabolic pathways and other processes such as flagellum synthesis and toxin production (17). The second messenger, cAMP, is synthesized from ATP by adenylate cyclase (168). Once bound to CRP, cAMP alters CRP's conformation into an active form that binds to a symmetrical DNA sequence with consensus of 5'-TGTGA-N_6-TCACA-3'. Typically, cAMP-CRP then interacts with RNA polymerase in one of multiple ways to modulate the initiation of transcription of target genes (77). As a global regulator, cAMP-CRP regulates over a hundred promoters in *E. coli* (20) leading to increased expression of transport systems and enzymes utilized in catabolizing various carbon sources, as well as enzymes in the tricarboxylic acid (TCA) cycle such as malate dehydrogenase, citrate

synthase, succinate dehydrogenase, and aconitase (30, 158, 165). Because of this role, CRP is alternatively known as CAP, for catabolite activator protein.

cAMP-CRP is best known for its role in catabolite repression in *E. coli*, in which the cell minimizes catabolism of other sugars in the presence of glucose. The phenomenon of diauxic growth occurs when a cell uses up the glucose and then moves on to catabolizing a non-preferred carbon source, such as lactose in the case of *E. coli* (58). In the presence of the preferred carbon source, glucose, the cellular cAMP levels are lowered to prevent transcriptional activation of catabolic genes needed for utilization of the non-preferred carbon sources (58). Glucose availability is connected to cAMP-CRP through the glucose phosphotransferase system (PTS) (Fig. 1.5) (58). During PTS transport of sugars, phosphoenolpyruvate (PEP) supplies the phosphoryl group to enzyme I (EI) which subsequently relays the phosphoryl group to the histidine protein (HPr) and then to cytoplasmic $EIIA^{Glc}$ domain and then to the membrane associated EIIB domain of the sugar transporter (58). When glucose is present, the phosphoryl group is ultimately relayed to glucose during import by the EIIC membrane domain, leaving $EIIA^{Glc}$ unphosphorylated.

The $EIIA^{Glc}$ protein and its phosphorylation state play an important role in catabolite repression in *E. coli* (9). In the absence of glucose, $EIIA^{Glc}$ is mainly in its phosphorylated form, but when glucose is available appreciably more $EIIA^{Glc}$ is unphosphorylated. This unphosphorylated $EIIA^{Glc}$ can bind and inactivate certain permeases and other proteins involved in the uptake and utilization of alternative carbon sources such as lactose through a mechanism called inducer exclusion, making this the primary mechanism of catabolite repression in *E. coli* (58). This observation and other studies illustrate that other regulatory mechanisms are present besides modulation of cAMP-CRP for catabolite repression (58).

Nonetheless, the regulation of adenylate cyclase (CyaA) activity (6, 9, 48, 119, 150) in response to the phosphorylation state of $EIIA^{Glc}$ connects glucose to this regulation in which the phosphorylated form of $EIIA^{Glc}$ interacts with CyaA to activate cAMP synthesis in the absence of glucose (9, 168). In *E. coli* it has been thought that intracellular cAMP is mainly controlled in response to glucose based on its synthesis by CyaA, which is regulated both post-translationally (123, 129), as noted above, or transcriptionally (2, 106). Turnover of cAMP is accomplished by a cAMP phosphodiesterase, CpdA, which breaks down cAMP into 5'-AMP. This enzyme was first partially purified in *E. coli* by Nielsen *et al.* (111), and later it was purified and characterized by Imamura *et al.* (67) and shown to be specific for the degradation of only cAMP and not cGMP like some other phosphodiesterases. It was confirmed that CpdA does degrade cAMP and that a *cpdA*-disrupted strain had two-fold higher cAMP than the parent strain (67).

Another means of regulating intracellular cAMP concentrations is through export (131). Hantke *et al.* speculated that in *E. coli,* export is the main mechanism for lowering intracellular cAMP levels, noting that CpdA has a low affinity for cAMP (61). Hantke *et al.* further postulated that TolC, an outer membrane channel protein, in conjunction with a cytoplasmic export system, is responsible for exporting cAMP out of the cell. However, this hypothesis was only assessed indirectly, by measuring *β*-galactosidase activity with the assumption that this activity directly correlates with cAMP levels (61). In addition after requesting strains from this group, I was told that the *tolC* mutant (H41) published in the paper was not actually a *tolC* mutant; however, *β*-galactosidase expression was more sensitive to low amounts of added cAMP with a correct *tolC* mutant, which they interpret as decreased cAMP export. Although regulation of cAMP export is not well described at this time, this mechanism is a valid phenomenon that could influence intracellular cAMP as shown in *E. coli* and other bacteria (131).

The evidence that cAMP levels are lowered in the presence of glucose through glucose phosphorylation by the PTS has been a widely accepted model in *E. coli* and in other systems (69, 94, 115, 117). It has been continually established that growth on glucose results in two- to five-fold lower intracellular cAMP in *E. coli* (115). Whether the regulatory effect of glucose and cAMP-CRP is mediated entirely by modulation of cAMP levels has been questioned. Ishizuka *et al.* saw a 2-fold decrease in cAMP after glucose was added to exponentially growing cells, but

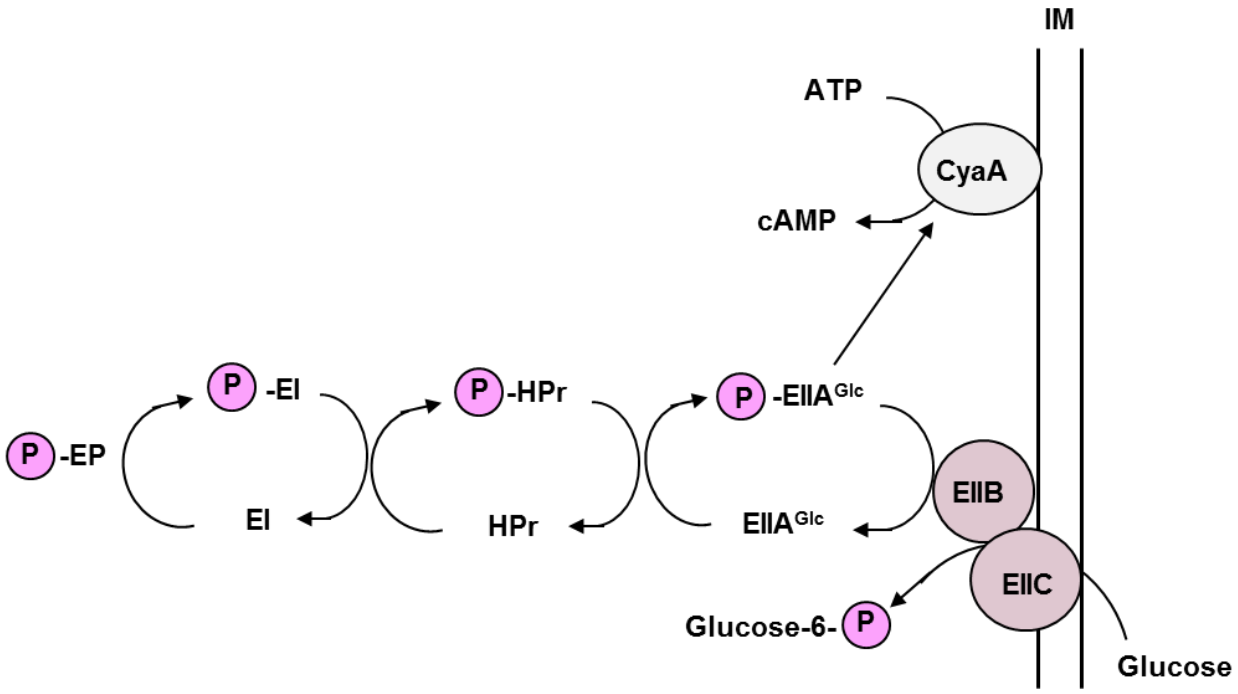

Figure 1.5: Phosphotransferase system in *E. coli*. In the presence of glucose, phosphoenolpyruvate (PEP) transfers a phosphate group to enzyme I (EI) then to the histidine protein (HPr) then to enzyme IIA cytoplasmic domain ($EIIA^{Glc}$) and then to enzyme IIB domain (EIIB) as glucose is transported through the enzyme IIC transporter (EIIC). Glucose is ultimately phosphorylated which results in less activation of adenylate cyclase (CyaA) to synthesize cAMP from ATP. In this model, the presence of glucose results in lowered cAMP levels.

suggested that the decrease in cAMP was too small to account for the significant decrease seen with β-galactosidase expression (69). Additionally, they found that high concentrations of exogenous cAMP did not eliminate catabolite repression by glucose, which suggested that

decreased cAMP alone is not enough to explain the repressive effect of glucose (69). They speculated that the level of CRP protein must be lowered as well, and tested this model by growing *E. coli* with or without glucose and measuring the amount of protein using an immunoblot analysis (69). There was a 2.5-fold reduction in the amount of CRP protein in the presence of glucose in several *E. coli* strains (69). To further test that the modulation of CRP rather than cAMP levels was critical to cAMP-CRP based regulation, *crp* was constitutively expressed on a plasmid to overcome catabolite repression in the presence of cAMP showing that glucose lowers both cAMP and CRP levels in *E. coli* (68).

The widely accepted model that phosphorylated $EIIA^{Glc}$ interacts with adenylate cyclase to stimulate cAMP synthesis has been largely based on mutant analysis. Park *et al.* took an *in vitro* approach and found that both the phosphorylated and unphosphorylated forms of $EIIA^{Glc}$ interact with the C-terminal domain of adenylate cyclase without stimulating cAMP synthesis (113). However, adenylate cyclase could be activated by the phosphorylated form of $EIIA^{Glc}$ only in the presence of *E. coli* crude cell extract suggesting the high activity of adenylate cyclase requires an additional unknown regulatory factor that is present in the crude extract (113). These data indicate that the mechanism(s) by which cAMP-CRP is controlled in *E. coli* in response to glucose remains an open question.

CRP-mediated regulation and its role in V. fischeri *luminescence*

Repression of luminescence in *V. fischeri* in response to glucose was first reported by Friedrich and Greenberg with strain MJ1, which is isolated from the light organ of the Japanese pinecone fish, *Monocentris japonica* (128). Later, it was reported that glucose does not affect luminescence in strain ES114, although the data and conditions were not shown (12). The

distinction between MJ1 and ES114 is important. They represent different clades within the *V. fischeri* species (16), their pheromone and luminescence systems appear to have been under selective pressures to diverge (16), and the symbiosis between ES114 and its host squid can be reconstituted in the laboratory (126, 141), thus making it now a more prominent research model than MJ1. I supervised an undergraduate researcher, Anne Weeks, who retested the response of strain ES114 and found that glucose can repress luminescence in ES114, while also confirming that exogenous supplementation with cAMP can increase luminescence of this strain. These observations were published in a paper that I co-authored (91).

To study the mechanism of catabolite repression of *V. fischeri* MJ1, Dunlap and Greenberg cloned the *lux* operon of MJ1 into *E. coli*. In this context too, glucose repressed luminescence, and this repression could be relieved by adding cAMP (37). The effects of glucose, cAMP, and autoinducer on luminescence and luciferase activity were studied in both the *E. coli* parent strain and in *crp* and *cya* mutants (37). They concluded that the induction of luminescence requires cAMP-CRP (37), and hypothesized that cAMP-CRP binds to the *luxR* promoter increasing *luxR* transcription, and that the increased pools of LuxR activate *luxICDABEG* (38). Dunlap *et al.* provided evidence to support this hypothesis by cloning *luxR* downstream of an IPTG-inducible promoter to produce constitutively high levels of LuxR in *cya* and *crp* mutants containing the *lux* operon and saw that the *cya* and *crp* mutations did not affect luminescence (38). Also, undefined *cya*- and *crp*-like mutants of *V. fischeri* MJ1 were isolated and showed similar results to transgenic *E. coli crp* and *cya* mutants with *lux* on recombinant plasmids (35).

These and other data suggested that CRP affects luminescence by regulating *luxR* transcription (38). In addition, a CRP binding site was identified in the *luxR-luxI* intergenic region based on similarity to the CRP binding site consensus sequence of *E. coli* (Fig. 1.6), and by a footprint analysis (38, 136). I co-authored a paper demonstrating, among other things, that while the LuxR-LuxI intergenic region has rapidly diverged between a variety of *V. fischeri* strains, the putative CRP-binding site has been largely conserved (16). It is possible that the spacing between the putative CRP box and the *luxR* promoter varies by 1-2 bases between strains, but the location of CRP binding with respect to the promoter remains uncertain, because, unfortunately, the *luxR* transcriptional start site has not been determined.

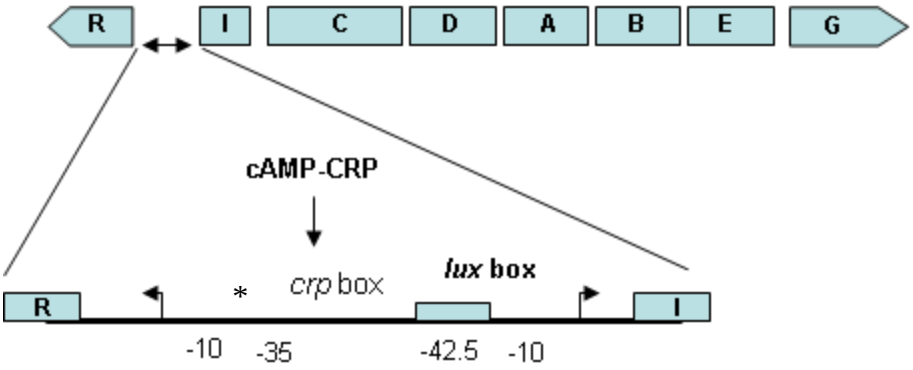

Figure 1.6: Location of *crp* box of the *lux* operon in *V. fischeri* MJ1. Model showing where cAMP-CRP may bind to facilitate RNA polymerase binding to the *luxR* promoter and increase transcription of *luxR.* * indicates that the transcriptional start site(s) has not been mapped in *V. fischeri*.

Similarly, work on the bioluminescent bacterium, *V. harveyi* showed that glucose repressed luminescence and implied a role for CRP in this control. Chatterjee *et al.* demonstrated that *V. harveyi* CRP binds directly to the *luxCDABE* promoter (this species lacks *luxI* and *luxG*) with the addition of cAMP, and that a *crp* null mutant resulted in elimination of luminescence (26). In transgenic *E. coli*, the *V. harveyi* CRP can functionally substitute in for *E. coli*'s CRP to activate *lux* (26).

Although CRP appears well conserved structurally and functionally between *E. coli* and the *Vibrionaceae*, sharing ~95% amino acid identity and the ability to cross complement, studies in transgenic *E. coli* can miss elements of *lux* regulation found in their native background. For example, work done with the *V. fischeri luxR-luxG* region cloned into *E. coli* would not have detected effects on the AinS-AinR system, which *E. coli* lacks. Lyell *et al.* found that cAMP-CRP regulates not only *luxR*, but also *ainS* in *V. fischeri* ES114, adding another layer of complexity to this pheromone-signaling network and underscoring the connection between cAMP-CRP and pheromone signaling (91).

The connections between glucose and pheromone-mediated signaling are not unique to *V. fischeri.* Glucose affects relatively unrelated systems, including peptide pheromone-signaling systems in Gram-positive bacteria, such as the *agr* system in *Staphylococcus aureus* (13). Moreover, in addition to *V. fischeri*, other Proteobacteria also specifically use cAMP-CRP to modulate pheromone signaling, and a few examples of cAMP-CRP control over pheromone systems will be outlined below.

CRP-mediated regulation of pheromones

As noted above, CRP is a global regulator, and when bound to cAMP, the cAMP-CRP complex binds DNA to either activate or inhibit transcription of target genes (77). CRP controls pheromone-mediated signaling in other systems, that regulate many diverse functions. As examples, I will describe other AHL and AI-2 signaling systems in *Pseudomonas aeruginosa*, *Erwinia chrysanthemi*, and *E. coli / Salmonella enterica.*

P. aeruginosa las and rhl systems and the effect of the CRP-homolog, Vfr

The opportunistic pathogen, *P. aeruginosa* uses AHL-based signaling through the *las* and *rhl* systems to regulate the production and release of extracellular virulence factors. Each of these systems resembles the LuxI-LuxR signaling system of *V. fischeri* but with distinct AHL signals and regulatory effects. The *las* system has a transcriptional activator, LasR that binds and responds to the pheromone 3-oxododecanoyl-homoserine lactone (3OC12) (32). The *rhl* system generates the AHL pheromone butyryl-homoserine lactone (C4), which functions through its cognate transcriptional activator RhlR (32). When bound to 3OC12, LasR becomes activated and promotes transcription of *rhlR*. RhlR will then combine with the other pheromone, C4, to activate the transcription of several genes including the *rhlAB* operon, which encodes proteins in rhamnolipid synthesis (29). LasR and RhlR belong to the LuxR family of transcriptional regulators and bind to sequences resembling the *lux*-box recognized by LuxR in *V. fischeri* (29, 163).

A CRP homolog was identified in *P. aeruginosa* and named Vfr, Virulence factor regulator, due to its role in virulence factor production (148). Vfr is involved in the transcriptional regulation of both of the transcriptional regulators of *P. aeruginosa*'s AHL systems, *lasR* (3, 148, 161) and *rhlR* (29). Several Vfr-binding sites were found in the *rhlR* promoter region (71), one of which represses transcription. Additionally, two of the binding sites overlap the *las* boxes where LasR/3OC12 binds to activate *rhlR* (29).

Vfr can substitute for CRP in *E. coli*; however, Vfr seems to play a different physiological role in *P. aeruginosa* (148). Vfr and *E. coli* CRP share 67% identity and 91% similarity and have a similar structure, but there are notable functional differences (135, 148). Serate *et al.* showed that Vfr's ligand sensing and response differs from *E. coli*'s CRP in that Vfr

has a significantly higher affinity for cAMP than CRP does, and Vfr can be activated not only by cAMP but also by cGMP (135). Moreover, in some cases, such as the regulation of *lasR*, Vfr activity does not require the binding of cAMP (53, 135). Although there are many similarities between CRP and Vfr, including the ability of *vfr* to restore catabolite repression of the *lac* promoter in an *E. coli crp* mutant, Vfr is not required for catabolite repression in *P. aeruginosa* (148). At this time, the exact link between environmental conditions, cAMP levels, and Vfr-mediated regulation in *P. aeruginosa* remains uncertain. Nonetheless, this CRP homolog clearly modulates AHL-mediated signaling.

Erwinia chrysanthemi expI-expR

E. chrysanthemi is a plant pathogen that causes soft-rot disease by degrading plant cell walls through the activity of extracellular enzymes such as pectinases. One network that modulates the expression of pectinases is pheromone-mediated regulation by ExpR and ExpI. ExpI, a homolog of LuxI, is an AHL synthase that makes both 3OC6 and *N*-hexanoyl-homoserine lactone (C6), although 3OC6 is the more abundant signal (109). ExpR, a homolog of LuxR, is convergently transcribed with ExpI and binds to the promoters of *expI*, *expR*, and target pectinase genes. ExpR has the greatest affinity for a conserved lux box-like sequence of its own promoter, and the position of this *lux* box-like site suggests repressor activity (24, 124). ExpR is a part of a LuxR-subfamily that differs from LuxR of *V. fischeri* in that they can dimerize, bind to DNA, and regulate target genes without binding their cognate AHL ligand (24). ExpR of *E. chrysanthemi* can regulate itself through AHL-independent repression and 3OC6-mediated de-repression (24). This regulation is cell density-dependent; at low cell density ExpR

auto-represses and once 3OC6 accumulates, de-repression at the *expR* promoter occurs in response to 3OC6 binding to ExpR (24).

Catabolite repression is observed in *E. chrysanthemi* during growth on glucose and in the presence of pectin catabolic products (125). *E. chrysanthemi*'s CRP shares 98% identity to *E. coli* CRP and can functionally replace CRP in *E. coli* (125). *E. chrysanthemi*'s CRP is responsible for positively or negatively regulating the *expR* and *expI* promoters, respectively (124). This connection is further illustrated by the increased levels of 3OC6 and C6 and a decrease in pectinase synthesis in a *crp* mutant (124, 125). Thus, in contrast to *V. fischeri* where both pheromone synthesis and the pheromone-responsive regulator are up-regulated by cAMP-CRP, in *E. chrysanthemi* pheromone synthesis and response are differentially regulated.

E. coli / S. enterica and AI-2 production

AI-2, the cross-species autoinducer, is found in many different bacteria and used to control a variety of processes. In *S. enterica* serovar Typhimurium, AI-2 (Fig 1.2) uptake and modification are performed by proteins encoded in the divergent *lsrACDBFGE* and *lsrRK* operons (167). LuxS synthesizes AI-2, which can accumulate outside of the cell but is transported into the cell through the Lsr ABC-type transporter. Upon entry, AI-2 is phosphorylated by LsrK (167). Phosphorylated AI-2 can either act as an inducer by binding to LsrR to relieve its repression of the *lsr* operon or become degraded by LsrF and LsrG (167). Accumulation of extracellular AI-2 occurs during exponential growth and rapidly decreases once the cells enter stationary phase due to the increase in AI-2 uptake by the Lsr transport system. The *lsr* operon in *E. coli* is both homologous to, and functions analogously to, the *S. enterica* serovar Typhimirium *lsr* operon (167).

Glucose affects AI-2 levels in both *Salmonella* and *E. coli*, and this effect is mediated by cAMP-CRP. Wang *et al.* showed that catabolite repression affects AI-2 accumulation through cAMP-CRP, which directly activates *lsr* transcription and indirectly represses *luxS* expression (159). In the presence of glucose, there is less cAMP-CRP to activate *lsr* transcription, resulting in less internalization of AI-2. The effect of glucose on AI-2 shows a link between catabolite repression and AI-2 levels through the cAMP-CRP regulation of AI-2 uptake, modification, and synthesis (167).

It is intriguing that different pheromone-signaling systems are each in some way controlled by CRP or similar proteins, and this observation suggests a connection between bacterial cell-cell communication and nutrition. Although a high cell density quorum may be required for signaling in each case, the control of pheromones in response to carbon source and availability could indicate a theme of group decision making with respect to nutrient scavenging. Having established clear links between cAMP-CRP and the Ain and Lux systems of *V. fischeri*, we sought to use this bacterium as a model to understand the connection between the environment, cAMP-CRP, and pheromone signaling. Ultimately, the ability to observe *V. fischeri* in natural symbiotic infections affords an opportunity to view such signaling in an ecologically relevant context; however, we first had to establish basic links between environmental conditions and modulation of cAMP-CRP.

Purpose of this research

Using the model of catabolite repression in *E. coli* (Fig 1.5), we predicted that the decrease of luminescence in *V. fischeri* grown on glucose is due to the reduced levels of cAMP. The PTS proteins of *V. fischeri* share a high degree of amino acid identity with those of *E. coli*;

ptsH (HPr, 79%), *pstI* (EI, 73%), and *crr* ($EIIA^{Glc}$, 83%) (4, 133, 155), and we therefore hypothesized a connection between $EIIA^{Glc}$ and cAMP levels similar to that reported in *E. coli*. On the other hand, Visick *et al.* saw that *ptsI* and *crr* mutants had increased luminescence compared to wild type suggesting that the PTS *inhibits* luminescence (155), which does not fit with a model in which $EIIA^{Glc}$ stimulates cAMP production resulting in cAMP-CRP stimulation of luminescence. The evidence outlined above also indicates that modulation of cAMP-CRP may be more complicated than what is portrayed in Fig 1.5, and that both the cAMP levels and CRP levels may be affected in the presence of glucose. We know that *V. fischeri* ES114 cAMP-CRP acts at the promoters of *luxR* and *ainS* to induce luminescence (91). However, it is still unclear how cAMP and CRP levels are regulated in *V. fischeri*. The mechanisms and responses to environmental cues remain unknown. I have sought to clarify in *V. fischeri* how glycerol and glucose affect the levels of intracellular cAMP and whether these levels may then affect CRP expression and regulation.

The initial aim of my research was to determine the importance of Δ*cyaA* and Δ*crp* in the symbiosis and growth on different carbon sources which is outlined in Chapter 2. In culture, Δ*crp* and Δ*cyaA* mutants have attenuated growth relative to wild type on all the carbon sources tested; however, growth relative to wild type is less attenuated when cells were provided glucose, *N*-acetylglucosamine, or, in the case of *cyaA* mutant, cAMP. This is consistent with the *E. coli* model in which there are low levels of cAMP and CRP in the presence of glucose. During symbiosis, the presence of cAMP and CRP are required for persistent colonization and needed for bright luminescence in culture.

In Chapter 3, I determined the intracellular cAMP levels during different growth conditions and in different mutant backgrounds, which better defined the mechanisms that

regulate cAMP levels. What I found is that cAMP levels are influenced by three mechanisms: synthesis, degradation, and excretion through proteins CyaA, CpdA/CpdP, and an unknown transporter, respectively. What still remains unclear is whether cAMP determines how cAMP-CRP activity is regulated. The data so far indicate that cAMP levels may not be the major factor influencing cAMP-CRP activity in *V. fischeri* when comparing cells grown on glycerol or glucose. Rather, I speculate there is a post-translational modification of CRP that alters its activity in *V. fischeri* grown on glucose.

Finally in the last chapter I will discuss how this research has contributed to the knowledge of cAMP-CRP regulation in Proteobacteria in addition to furthering our understanding of the controls governing bioluminescence and pheromone regulation by an environmental cue such as carbon source availability in *V. fischeri* ES114.

CHAPTER 2

THE IMPORTANCE OF CRP AND CYAA IN HOST COLONIZATION DURING THE *VIBRIO FISCHERI-EUPRYMNA SCOLOPES* SQUID SYMBIOSIS[1]

[1]Deanna M. Colton, Julie L. Stoudenmire, and Eric V. Stabb. To be submitted to *FEMS Microbiology Letters.*

Abstract

CRP, together with second messenger cAMP generated by CyaA, transcriptionally controls a broad regulon in many Proteobacteria and typically induces expression of genes when glucose is unavailable. In the light-organ symbiont *Vibrio fischeri*, symbiotic luminescence is induced by the AinS/AinR and LuxI/LuxR pheromone systems and transcription of both *ainSR* and *luxR* are activated by cAMP-CRP. Here, we examined the role of cAMP and CRP during *V. fischeri's* growth in culture and during symbiotic infection of its host squid, *Euprymna scolopes*. Both Δ*crp* and Δ*cyaA* mutants colonized the *E. scolopes* light organ poorly, achieving 1000-fold lower levels of colonization than wild type after 48 hours. Moreover, a cAMP-CRP-dependent reporter was expressed during the symbiosis, indicating activation of the CRP regulon during infection. In culture, Δ*crp* and Δ*cyaA* mutants displayed growth rates less than half of ES114's on most carbon sources. However, growth rates closer to those of wild type were achieved with glucose and *N*-acetylglucosamine, and cAMP completely restored growth of the Δ*cyaA* mutant. Our results provide insight into the nutritional status of *V. fischeri* in this light-organ symbiosis.

Introduction

Vibrio fischeri is a bioluminescent Proteobacterium that forms light-organ symbioses with certain marine animals, including the Hawaiian bobtail squid, *Euprymna scolopes*. The *V. fischeri-E. scolopes* symbiosis is a powerful model system for studying mutualistic symbioses because of the genetic tractability of *V. fischeri* and the ability to reconstitute this symbiosis in the laboratory (126, 141). Gene regulation during infection and the induction of symbiotic bioluminescence have been areas of particular interest, partly because bioluminescence is governed by pheromone-mediated regulation. In *V. fischeri* strain ES114, an isolate from *E.*

scolopes that serves as the wild type in many studies, bioluminescence is much more strongly induced during host colonization than during growth in culture (12). Studying regulators of bioluminescence can therefore provide valuable insight into the light-organ environment.

Among the first regulators of luminescence discovered was cAMP-CRP. Initially, Dunlap and Greenberg observed that cAMP-CRP controlled bioluminescence in transgenic *Escherichia coli* carrying the light-generating *luxR-luxICDABEG* region from *V. fischeri* (35, 37). More recently, we found that cAMP-CRP regulates the AinS/AinR and LuxI/LuxR pheromone systems, both of which coordinate regulation of luminescence (91).

Luminescence is a colonization factor for *V. fischeri* (15, 153), and in other bacteria the CRP regulon includes genes important for host infection, colonization, and virulence. For example, in *Vibrio cholerae*, cAMP-CRP controls the quorum sensing master regulator HapR, biofilm formation, motility, and host colonization (21, 50, 84-86). *V. cholerae crp* mutants produce elevated cholera toxin and toxin co-regulated pilus, yet they are also defective in mouse intestinal colonization, showing the importance of CRP for survival in the host (140).

In addition to controlling host-associated phenotypes, the cAMP and CRP duo is best known in Proteobacteria for activating catabolic genes required for utilization of non-glucose carbon sources. To accomplish this regulatory role, adenylate cyclase (CyaA) generates the second messenger cAMP, which binds to CRP and alters its conformation, enabling CRP to bind DNA and activate, or less frequently to inhibit, transcription at target promoters (77). cAMP-CRP interacts with RNA polymerase (and potentially other regulators) in Class I, II, or III type interactions (20). In each case CRP binds to a 22-bp repeat with the core consensus motif 5'-TGTGA-N6-TCACA-3' (20, 77). During growth on a preferred substrate such as glucose, there is generally less cAMP-CRP activity, either through decreased levels of cAMP, modulation of

CRP, or both (9, 58, 69). For example, in *E. coli*, the $EIIA^{Glc}$ component of the glucose phosphotransferase system (PTS) affects CyaA activity in such a way that glucose availability leads to decreased cAMP levels and lower cAMP-CRP activity (100, 121).

Carbon source availability is a defining feature of the host environment, but the growth substrates that *V. fischeri* utilizes and the availability of preferred substrates in its symbioses are not well understood. Nealson and Ruby suggested that glucose metabolism might play a symbiotic role in fish light organs (127), but the hosts were not experimentally tractable. In the *E. scolopes* symbiosis, host epithelial cells slough into the light-organ crypts, producing a complex growth medium (126). Moreover, *V. fischeri* can access amino acids, mostly in the form of peptides during colonization (59), and may utilize sugars such as mannose that decorate host cell surface molecules (98). Wier *et al.* found metabolic patterns in the symbionts that followed the squid's diurnal cycle to suggest that chitin, *N*-acetylglucosamine (NAG), glycerol, and glycerol-3-phosphate are utilized during the symbiosis (164); however, a *nagB* mutant impaired in NAG utilization showed no apparent symbiotic defect (103). In contrast, a *ptsI* mutant affected in the utilization of several fermentable sugars, including glucose, was severely impaired in colonization (1). Despite what is known, it is difficult to predict the activity and importance of cAMP-CRP in the *V. fischeri*-*E. scolopes* symbiosis, and we therefore addressed these issues in the present study.

Table 2.1: Bacterial strains and plasmids used in this study

Strain and plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(60)
DH5α*λpir*	DH5α lysogenized with *λpir*	(39)
CC118*λpir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA λpir*	(145)
V. fischeri		
ES114	Wild-type isolate from *E. scolopes*	(12)
JB24	ES114 Δ*crp*	(14)
DC03	ES114 Δ*cyaA*	This study
DC18	ES114 Δ*cpdP*	This study
DC48	ES114 Δ*cpdP* Δ*cpdA*	This study
DC55	ES114 Δ*cpdP* Δ*cpdA* Δ*cyaA*	This study
Plasmids[b]		
pAH9	*sarA* promoter P1-*mCherry* (RFP) *ampR*, *ermR*	(13)
pDC5b	Δ*cyaA* allele; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *ermR*	This study
pDC40	Δ*cpdP* allele; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *camR*	This study
pDC54	Δ*cpdA* allele; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *camR*	This study
pDC85	*oriV*R6Kγ, $oriT_{RP4,}$ pES213, *mCherry*, *kanR*, $P_{CRP\text{-}D}$-*camR-gfp*	This study
pEVS104	conjugative helper plasmid; *oriV*R6Kγ, $oriT_{RP4}$, *kanR*	(145)
pJLS27	*oriV*R6Kγ, $oriT_{RP4,}$ pES213, *mCherry*, *kanR*, promoterless-*camR-gfp*	This study
pVSV33	*oriV*R6Kγ, $oriT_{RP4,}$ pES213, *kanR*, promoterless-*camR-gfp*	(40)
Oligonucleotides		

[c]		
CRPartpromF[d]	GAT GCA TGC TAA TGT GAG TTA GCT CAC TCA T	This study
CRPartpromR	TGA GTC GAC AGN NNN NNA GTG TAN NNN NNN NNN NNNNAT GAG TGA GCT AAC TCA CAT TAG CAT GCA TC	This study
cyaAupF3	GGC GCG TGT GCT CAA CAG CGT GCT GAC	This study
cyaAupR3	ATG GGC GCG CCC ATT AGT GCT AAA GCT CGC TCT ACA CG	This study
cyaAdnF2	ATG GGC GCG CCC TAA AAA GCA AAA AGA GAT CCG AGA TTT GGG	This study
cyaAdnR2	GGC CGC CTT TCT CTT GTT CAT ACA TAG CAA AC	This study
Clalprimer3	CGC GAA AAT CGA TAA A	This study
Cla1primer4	CGC GTT TAT CGA TTT T	This study
cpdPoligoA	CTT TTA ATC CCC AAT AAA CCA TGT AGG	This study
cpdPoligoB	GCT AGC CAT ATC CAA CCT TAA ATA ATA	This study
cpdPoligoC	TAT TAT TTA AGG TTG GAT ATG GCT AGC TAA AAA AGT TAT TGT AAA AAT	This study
cpdPoligoD	GGT CAA GTT ATA GAG TGG AAT CAT ATT CTT G	This study
cpdAoligoA	CGA TCA TTA TCA CGA GTA TCG TTG TCA C	This study
cpdAoligoB	GCT AGC CAA CCT TTT AAT TCT TTT CTA AAA	This study
cpdAoligoC	TTT TAG AAA AGA ATT AAA AGG TTG GCT AGC TAA TGA ATA AAC CAA	This study
cpdAoligoD	CGT GCG TAC CAC CTT GTG CT	This study
JLSmcherryF3	TAC GGC CGT TGA CAT AAA GTC GAA GTT ATT CTA TAA TAG GAT CCC CGG GTA CCT AGG GAG	This study
JLSmcherryR	GCG CGG CCG TTA CTT GTA CAG CTC GTC CAT GCC	This study

[a] Drug resistance abbreviations used: *camR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance; *ampR*, ampicillin resistance

[b] All alleles cloned in this study are from *V. fischeri* strain ES114. Replication origin(s) of each vector are listed as R6Kγ, ColE1, and/or pES213. Plasmids based on pES213 are stable in *V. fischeri* ES114 and do not require antibiotic selection for maintenance (40).

[c] All oligonucleotides are shown 5' to 3'. Underlined regions highlight restriction enzyme recognition sites.

[d] "N" indicates randomized bases

Materials and Methods

Growth media and reagents

Escherichia coli cultures were grown at 37ºC in LB medium (101) with final concentrations of 20 μg ml^{-1} chloramphenicol (cam), 40 μg ml^{-1} kanamycin (kan), or 60 μg ml^{-1} ampicillin (amp) when appropriate for selection. *V. fischeri* was grown in either LBS medium (144) , SWTO medium (14), ASWT medium (12) where seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, OH), or supplemented fischeri mineral salts medium (FMM) (134) containing either 40 mM glycerol, 20 mM glucose, 16.7 mM *N*-acetylglucosamine, 20 mM fructose, 20 mM mannose, or 10 mM cellobiose as indicated. For selection in *V. fischeri*, 2 μg ml^{-1} chloramphenicol (cam) or 5 μg ml^{-1} erythromycin (erm) were added to LBS. Adenosine 3', 5'-cyclic monophosphate (cAMP) was obtained from Sigma-Aldrich (St. Louis, MO). Bromocresol purple sodium salt was obtained from Eastman (Rochester, NY) and prepared as a 1.5% w/v solution for use as pH indicator.

Strains, oligonucleotides, and plasmids

Strains, plasmids, and oligonucleotides used in this study are listed in Table 2.1. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). *V. fischeri* ES114 was the wild-type strain used (12). *E. coli* strains DH5α (60) or DH5αλpir (39) were used for the cloning with the latter used to maintain plasmids bearing the R6K origin of replication. Mutant alleles were transferred from *E. coli* into *V. fischeri* on plasmids by triparental mating using the conjugative helper plasmid pEVS104 (145) in strain CC118λ*pir* (64).

Molecular genetics and sequence analyses

Plasmids were constructed using standard techniques as described previously (76). In-frame deletion mutants were made through allelic exchange (15) and verified by PCR. The resulting mutants are listed in Table 2.1. To generate a mutant lacking *cyaA* (VF0067), we generated a deletion allele consisting of 1.5 kb upstream of *cyaA* (amplified with primers cyaAupF3 and cyaAupR3) fused at an *Asc*I site to 1.5 kb of downstream of *cyaA* (amplified with primers cyaAdnF2 and cyaAdnR2), and we inserted a small dsDNA fragment consisting of oligos ClaIprimer3 and ClaIprimer4 at the *Asc*I site to restore the reading frame, resulting in the sequence 5'-GGC GCG AAA ATC GAT AAA CGC GCC-3' between the *cyaA* start and stop codons. The resultant plasmid, pDC5b, was moved into ES114 for allelic exchange, resulting in the in-frame deletion mutant DC03 (Table 2.1).

To generate a mutant lacking *cpdP* (VF1256), a deletion allele was made using overlap extension PCR (SOE-PCR) (65) in which 1.5 kb upstream of *cpdP* (amplified with primers cpdPoligoA and cpdPoligoB) was connected to the 1.5 kb downstream of *cpdP* (amplified with

primers cpdPoligoC and cpdPoligoD) by complementary sequences at the 3'-end of cpdPoligoB and 5'-end of cpdPoligoC. The resultant plasmid, pDC40, contained the start and stop codons of *cpdP* separated by a *Nhe*I site and was moved into wild-type ES114 for allelic exchange to make DC18 (Table 2.1). To generate a deletion allele for *cpdA* (VF2230), 1.5 kb upstream of *cpdA* (amplified with primers cpdAoligoA and cpdAoligoB) was fused at a *Nhe*I site to 1.5 kb downstream of *cpdA* (amplified with primers cpdPoligoC and cpdPoligoD). The resultant plasmid, pDC54, was then moved into DC18 to make the Δ*cpdP* Δ*cpdA* double mutant DC48. We then moved the Δ*cyaA* allele on pDC5b into DC48 to make the Δ*cpdP* Δ*cpdA* Δ*cyaA* mutant, DC55.

To generate the reporter parent vector pJLS27, mCherry was PCR amplified from pAH9 using primers JLSmcherryF3 and JLSmcherryR (Table 2.1). JLSmcherryF3 added a promoter upstream of the mCherry ribosome binding site. The resultant PCR product was digested with *Eag*I and cloned into *Eag*I-digested pVSV33, yielding a constitutive mCherry separated by transcriptional terminators from a promoterless *cat-gfp* reporter.

Construction of the artificial cAMP-CRP-dependent Class II promoter ($P_{CRP\text{-}D}$) reporter plasmid pDC85 is described in greater detail below. Briefly, oligos CRPartpromF and CRPartpromR were annealed together and DNA polymerase I (Klenow fragment) from New England Biolabs was used to fill in single-stranded overhangs. The fill-in products were digested with *Sal*I and *Sph*I and cloned upstream of *cat-gfp* in pJLS27 between the *Sal*I and *Sph*I sites. Fluorescence intensity of strains carrying the resultant plasmids or pJLS27 was measured using a Synergy 2 plate reader (Biotek, Winooski, VT). GFP and mCherry fluorescence were measured using excitation/emission wavelength pairs of 485 nm/528 nm and 530 nm/590 nm,

respectively. Green fluorescence was normalized to constitutive red fluorescence of mCherry to determine the specific fluorescence (GFP/mCherry).

Squid colonization assays and microscopy

For squid colonization assays, *V. fischeri* strains were grown in ASWT at 28ºC without shaking until an OD_{595} of 0.4 to 0.7. Inocula were diluted in filtered Instant Ocean and dilution plated onto LBS to determine CFU ml^{-1}. Newly hatched juvenile *E. scolopes* were placed in the inocula for up to 20 hours and then moved to inocula-free Instant Ocean. After 24 or 48 hours, squid were homogenized and plated on LBS to determine CFU/squid. To assess fluorescence of transgenic *V. fischeri* symbionts with pDC85 or pJLS27, squid were dissected and viewed by epifluorescence microscopy using a Nikon Eclipse E600 microscope. In parallel, strains with pDC85 or pJLS27 were grown on SWTO agar plates supplemented with either glycerol, glucose, NAG, or mannose and grown overnight at 28°C, at which point colonies were likewise imaged. A Nikon 51004v2 filter set was used to visualize the constitutive red fluorescence along with the green fluorescent reporter simultaneously.

Results

CyaA and Crp are important for host colonization

To assess cAMP-CRP's role during the squid symbiosis and determine whether *cyaA* and *crp* are important for host colonization, we assayed the ability of the Δ*cyaA* and Δ*crp* mutants to colonize juvenile squid. In our first experiment, at a comparable inoculum around 2000 CFU ml^{-1}, squid colonization was undetectable with a Δ*crp* mutant as opposed to the squid colonized by ES114. When the Δ*crp* mutant inoculum was increased ~5-fold more than wild type, squid

were colonized yet at 200-fold less CFU/squid than wild type at 24 hours and over 1000-fold less than wild type at 48 hours (Fig. 2.1A). We saw similar colonization defects with the Δ*cyaA* mutant in another experiment. Figure 2.1B shows one typical experiment with the Δ*cyaA* mutant where at an inoculum level around 2000 CFU ml^{-1} only three out of the eleven animals were colonized, resulting in over 1000-fold less CFU/squid than wild type. Thus both *cyaA* and *crp* are apparently required for robust colonization initiation and persistence.

Poor growth of ΔcyaA and Δcrp mutants on different carbon sources

To place the importance of *cyaA* and *crp* in *V. fischeri*'s symbiotic colonization in perspective, we wanted to determine the growth of these mutants in culture with different carbon sources. We compared both the growth rate (gen hr^{-1}) and growth yield of the Δ*crp* and Δ*cyaA* mutants relative to wild-type ES114, both in a complex medium (SWTO) and in a more defined medium (FMM) each amended with different carbon sources. We reported previously that the Δ*crp* mutant, JB24, was very dim and grew poorly (91). We wanted to see if this was also true for the Δ*cyaA* mutant, which cannot make the cAMP needed to activate CRP. As we predicted, the Δ*cyaA* mutant mimicked the Δ*crp* mutant in slow growth (Table 2.2) and dim luminescence (not shown). Exogenous cAMP reversed these defects for the Δ*cyaA* mutant but not for the Δ*crp* mutant (Table 2.2). Both the Δ*cyaA* and Δ*crp* mutants grew poorly on all the carbon sources tested (Table 2.2), with growth rates less than half that achieved by wild-type ES114. Compared to glycerol and mannose, glucose and *N*-acetylglucosamine (NAG) yielded substantially better growth rates for the Δ*cyaA* and Δ*crp* mutants relative to ES114. Moreover, on glucose, NAG, and fructose the Δ*crp* and Δ*cyaA* mutants eventually reached the same growth yields as ES114,

whereas on mannose, cellobiose, and glycerol the mutants' growth yields remained well under half of ES114's growth yield.

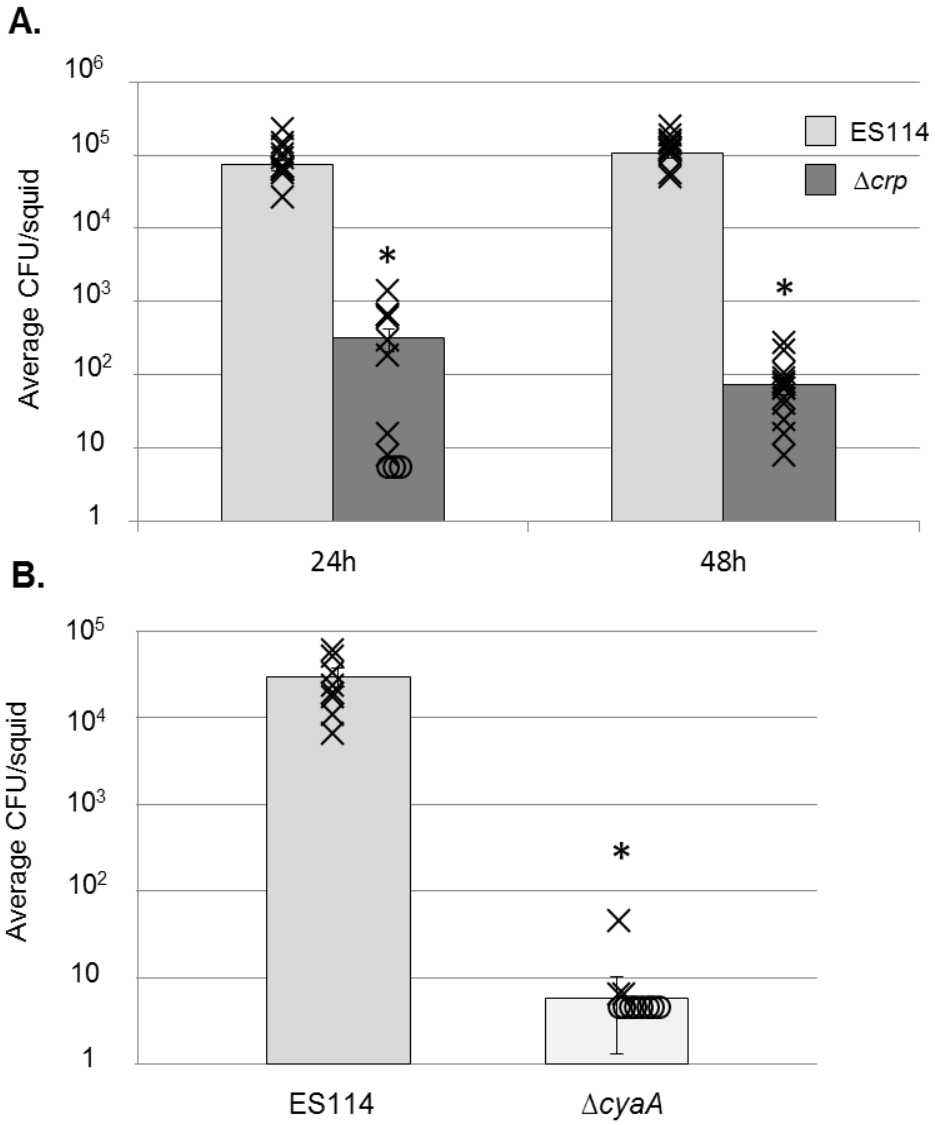

Figure 2.1: Symbiotic colonization by ES114, Δ*crp*, and Δ*cyaA* mutants. **(A)** 24 and 48 hours post colonization; ES114 inoculum, 1700 CFU ml^{-1}; Δ*crp* inoculum, 6500 CFU ml^{-1} (n =12-13). **(B)** 48 hours post colonization; ES114 inoculum, 2800 CFU ml^{-1}; Δ*cyaA* inoculum, 2000 CFU ml^{-1} (n = 10-11). Asterisks indicate $p< 0.05$. O's indicate squid with colonization below the limit of detection. X's indicate the number of *V. fischeri* cells in an individual squid, and the bar indicates the average colonization level.

Table 2.2: Growth rates and yields of ES114, JB24, and DC03 grown on different carbon sources

	Growth Rate (gen hr^{-1})[a]			**Growth Yield**[b]		
SWTO + Sugar	**ES114**	**JB24 Δ*crp***	**DC03 Δ*cyaA***	**ES114**	**JB24 Δ*crp***	**DC03 Δ*cyaA***
Glucose	1.6	0.8	0.9	6.8	10	9.8
NAG	1.7	0.8	0.8	8.9	7.5	8.3
Fructose	1.4	0.5	0.5	7.9	9.9	9.9
Mannose	1.4	0.3	0.3	7.3	1.6	1.4
Cellobiose	1.2	0.3	0.2	8.3	1.6	1.6
Glycerol	1.0	0.3	0.3	8.5	1.2	1.0
Glycerol + 5 mM cAMP	1.3	0.5	1.3	9.2	2.5	7.9
Glucose + 5 mM cAMP	1.0	0.7	1.1	8.6	10	7.5
FMM + Sugar						
Glucose	1.2	0.6	0.6	2.2	2.0	2.0
NAG	1.0	0.6	0.7	2.0	2.0	2.0
Glycerol	1.0	0.2	0.3	2.4	1.5	1.5
Glycerol + 5 mM cAMP	1.0	0.1	0.5	3.4	0.7	3.7
Glucose + 5 mM cAMP	1.1	0.8	0.9	2.1	2.0	2.0

[a] Growth rates calculated at OD_{595} 0.3-0.6

[b] Growth yield presented as the final OD_{595}

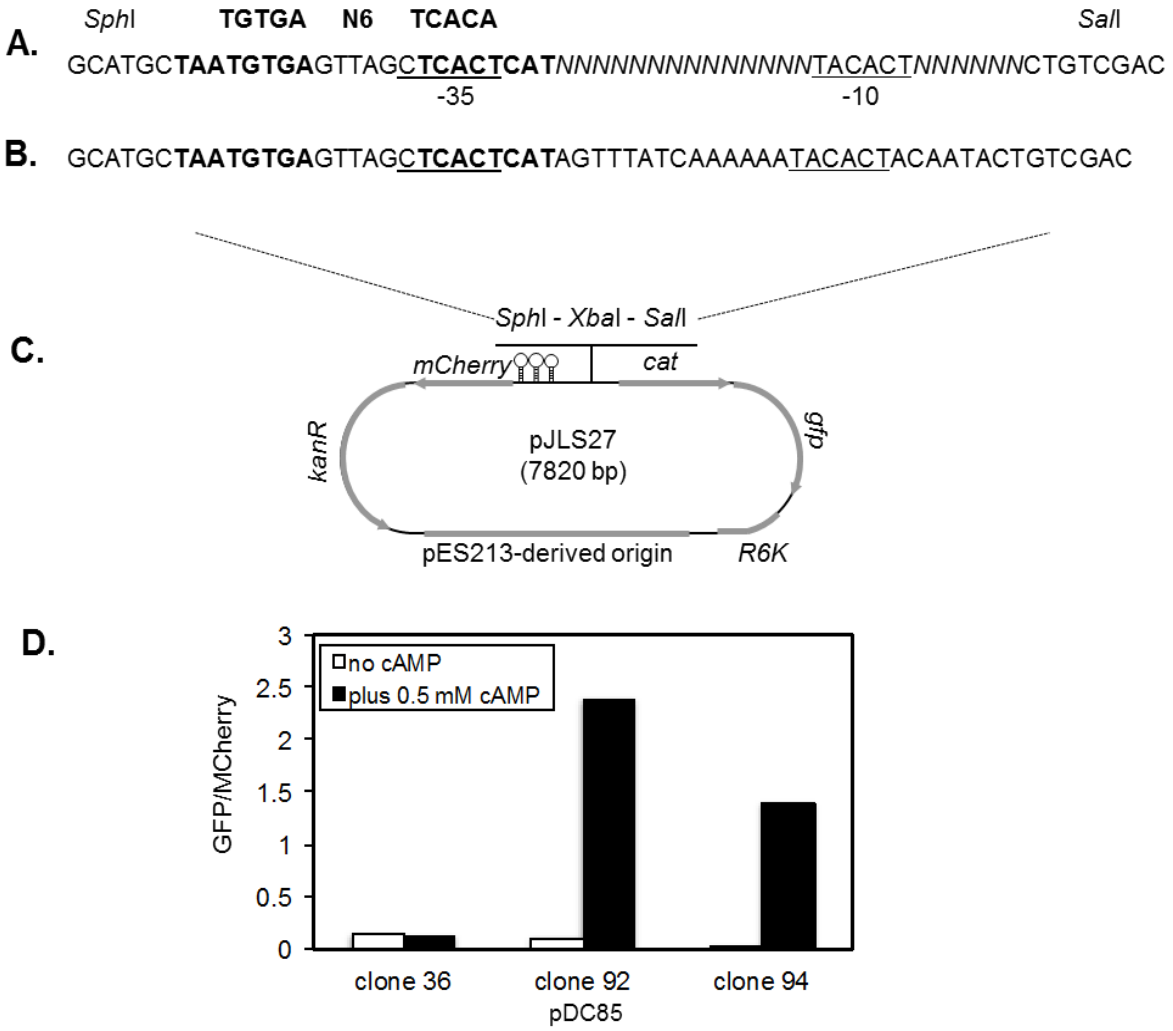

Figure 2.2: Construction and identification of $P_{CRP\text{-}D}$-*cat-gfp* reporter plasmid pDC85. Panel **(A)** shows engineered inserts with a Class II CRP-dependent promoter overlapping the -35 element that is based on the *E. coli lac* promoter but without the Lac operator and with many nucleotides randomized. The -10 and -35 promoter elements are underlined, and the CRP binding site is in bold, with the core 5'-TGTGA(N6)TCACA-3' indicated above. Panel **(B)** shows the insert sequence in pDC85. Panel **(C)** illustrates a map of parent reporter plasmid pJLS27, with a constitutive mCherry separated by three Rho-independent transcriptional terminators (indicated as stem loops) from a multiple cloning site and a promoterless *cat-gfp* reporter. Panel **(D)** shows results of three plasmids screened in DC55 (Δ*cpdP* Δ*cpdA* Δ*cyaA*) for responsiveness to cAMP, with green fluorescence normalized to the constitutive red fluorescence at culture OD_{595} between 0.4 and 0.5, and the fluorescence of pJLS27 subtracted as background. The plasmid in clone 92 was designated pDC85 ($P_{CRP\text{-}D}$-*cat-gfp* reporter) for further experiments.

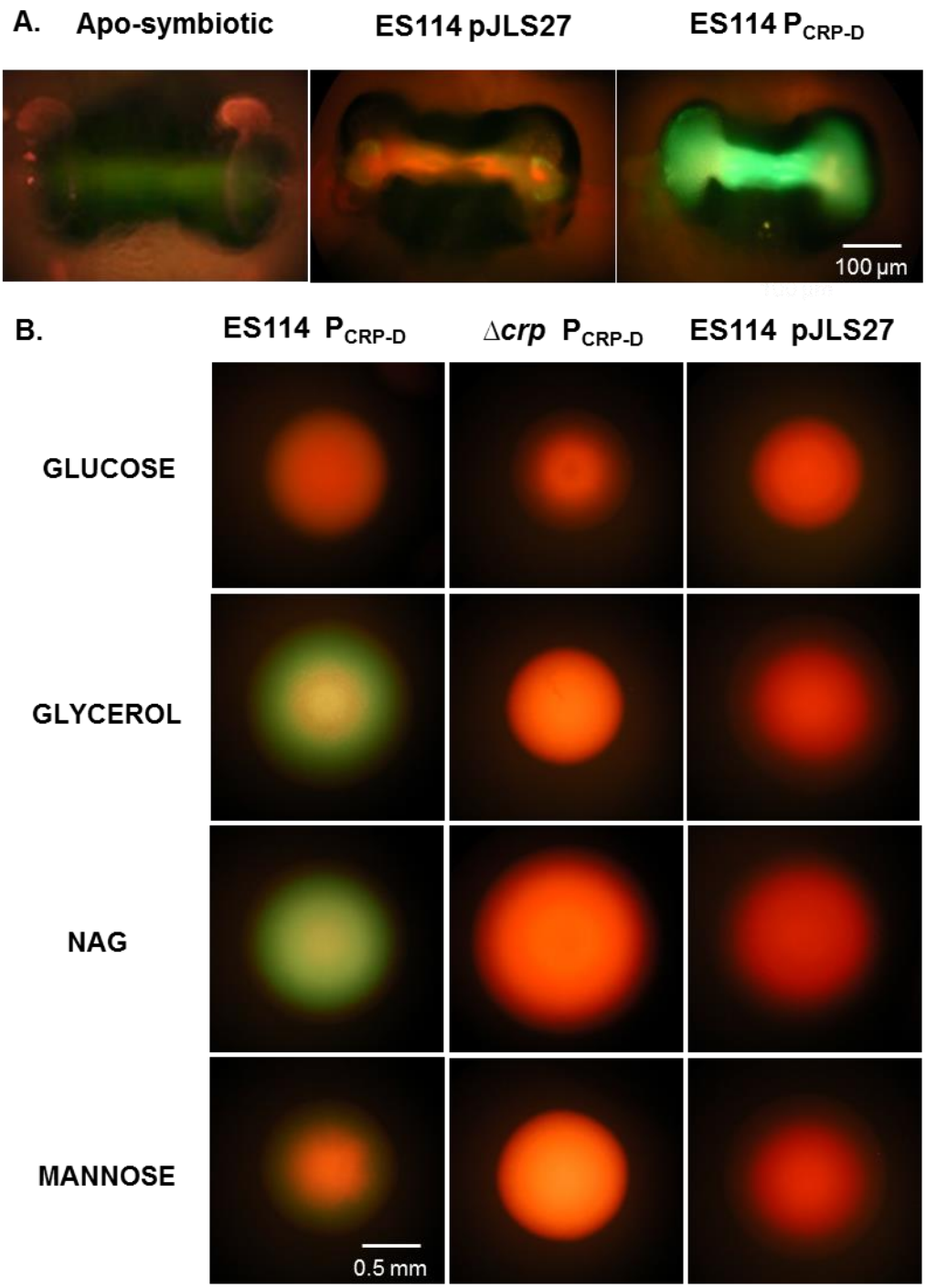

Figure 2.3: Epifluorescence images of *E. scolopes* light-organ and *V. fischeri* colonies. **(A)** Light organs from squid that were un-colonized or "apo-symbiotic", colonized by ES114 with promoterless-*gfp* vector pJLS27, and colonized by ES114 with P_{CRP-D}-*gfp* 48 hours post inoculation. **(B)** *V. fischeri* colonies grown on SWTO agar supplemented with indicated sugar. P_{CRP-D} in ES114 (left) and Δ*crp* (middle) compared to promoterless parent vector pJLS27 in ES114 (right).

A cAMP-CRP-dependent reporter is activated in V. fischeri symbionts

Interpreting the phenotypes of Δ*crp* and Δ*cyaA* mutants is complicated due to their poor growth under various conditions, so in order to investigate cAMP-CRP-mediated expression further we developed a *gfp* transcriptional reporter under the control of a cAMP-CRP-dependent promoter ($P_{CRP\text{-}D}$). Other studies have used CRP-dependent reporters to assess cAMP-CRP activity, but these have been based on native promoters that are also controlled by other regulators. We sought to generate an artificial promoter devoid of other known regulatory elements and we used the *lac* promoter sequence of *E. coli* as a template. A cAMP-CRP dependent promoter was designed with a strong consensus CRP site overlapping a weaker -35 element and a strong -10 site and randomized bases "N" in between (Fig. 2.2A) to remove non-CRP regulatory sites like the *lac* operator. Variants of this template were then cloned into pJLS27 (Fig. 2.2C) and *E. coli* transformants were screened for higher GFP/mCherry fluorescence relative to that of the promoterless parent vector. The best candidates were then moved into *V. fischeri* DC55 (Δ*cpdP* Δ*cpdA* Δ*cyaA*), which cannot make its own cAMP (our unpublished data), and screened for induced fluorescence with the addition of exogenous cAMP. Figure 2.2D illustrates three typical variants that were screened, with one clone showing no responsiveness to cAMP and two others that do. The plasmid in clone number 92 (Fig. 2.2D) was named pDC85, the promoter was sequenced and designated $P_{CRP\text{-}D}$ (Fig. 2.2B), and this reporter was used for further experiments.

The $P_{CRP\text{-}D}$-*gfp* reporter (pDC85) became a useful tool to monitor cAMP-CRP activity in both the squid and in culture. To test further whether cAMP-CRP-mediated expression is relevant in the symbiosis, juvenile squid were infected with ES114 carrying pDC85 or pJLS27 and dissected after 24 or 48 hours to visualize red and green fluorescence from the plasmid in

their light organs. Compared to apo-symbiotic squid or squid infected by ES114 with the promoterless control, the $P_{CRP\text{-}D}$-*gfp* reporter showed elevated green fluorescence suggesting cAMP-CRP activity in the light organ 24 hours (data not shown) and 48 hours after infection (Fig. 2.3A).

We also observed this reporter in *V. fischeri* strains grown on different carbon sources. To test whether this $P_{CRP\text{-}D}$-*gfp* is dependent on CRP, we moved it into the Δ*crp* mutant and examined colonies grown on media supplemented with glucose, NAG, glycerol, and mannose confirming that the CRP-dependent reporter is not active in the absence of *crp*, illustrated by the red fluorescence of the constitutive mCherry and lack of green fluorescence (Fig. 2.3B). Similar results were found with the promoterless parent vector in ES114 (Fig. 2.3B). In Table 2.2, we showed that both glucose and NAG improved the growth of both the Δ*cyaA* and Δ*crp* mutants, suggesting that cAMP-CRP-mediated regulation is not required for catabolism of these carbon sources. However, high green fluorescence was observed from the $P_{CRP\text{-}D}$-*gfp* reporter in ES114 during growth on NAG, but no with colonies grown with glucose (Fig. 2.3B). In addition, relatively little green fluorescence was observed in cells growing with mannose (Fig. 2.3B).

Discussion

In this study, we investigated the importance of cAMP-CRP in *V. fischeri*, particularly during colonization of the *E. scolopes* light organ. In other bacteria, CRP requires the second messenger cAMP to become an active regulator (77), and the *V. fischeri* genome indicates a single adenylate cyclase, CyaA, for production of cAMP. We therefore predicted that Δ*crp* and Δ*cyaA* mutants would behave similarly, which proved to be the case. The one notable and predictable difference between these strains was that amending the medium with cAMP restored

wild-type-like growth to the Δ*cyaA* mutant but not to the Δ*crp* mutant (Table 2.2). This observation seems to confirm what had been previously inferred (35, 91), that exogenous cAMP can enter *V. fischeri* cells at least to some extent.

Both the Δ*crp* and Δ*cyaA* mutants displayed very poor colonization of *E. scolopes*. The reason for this attenuation is unknown but may be multi-factorial, given that cAMP-CRP regulates many different functions. Among the known cAMP-CRP-controlled genes in *V. fischeri* are *luxR* and *ainSR* (91), which are involved in pheromone-mediated signaling and bioluminescence (142). The Δ*crp* (91) and Δ*cyaA* mutants are dim, and luminescence is a colonization factor required for symbiotic persistence (15, 153); however, weak luminescence alone cannot account for the colonization defect of the Δ*crp* and Δ*cyaA* mutants. A dark Δ*luxCDABEG* mutant is only attenuated three- to four-fold 48h after infection (15), whereas the Δ*crp* and Δ*cyaA* mutants were far more attenuated, close to 1000-fold, at the same time point (Fig 2.1). The LuxR and AinS/AinR regulons extend beyond luminescence (7, 23, 88, 122, 147), but the symbiotic defects of *luxR* and *ainS* mutants are also not as severe as those of the Δ*crp* and Δ*cyaA* mutants (87, 153). In other bacteria, attenuated virulence and/or host colonization have been reported for *crp* mutants (84, 85). It is likely that in *V. fischeri* CRP affects genes associated with colonization in addition to catabolic operons. Further investigation of the CRP regulon in *V. fischeri* may reveal other important factors in its symbiotic lifestyle.

To help interpret the symbiotic defects of the Δ*crp* and Δ*cyaA* mutants and put them in perspective, we also examined growth of these mutants cultured outside of the host with different growth substrates. One could argue that the poor colonization of the Δ*crp* and Δ*cyaA* mutants is due to their inability to grow on the present substrates in the light organ rather than a symbiosis-specific effect. It is true that Δ*crp* and Δ*cyaA* mutants showed growth defects in all conditions

tested, with the possible exception of the Δ*cyaA* mutant growing in certain media supplemented with cAMP (Table 2.2); however, growth rates and growth yields of the mutants in culture (Table 2.2) are not starkly different from wild type compared to their symbiotic defects. Growth yields were similar for the mutants and wild type under several culture conditions, and growth rates were modestly compromised when the cultured cells were provided glucose or NAG. It is tempting to speculate that neither, glucose, NAG, nor cAMP are major carbon sources for *V. fischeri* in the light organ, given that these compounds largely recover growth of one or both of the mutants in culture. Although such a model is consistent with our results, it is also possible that CRP regulates a colonization factor that is irrelevant to cultured cells to account for our data.

As an alternative and complementary approach to assessing the role of cAMP-CRP during symbiosis, we used a fluorescent cAMP-CRP-dependent transcriptional reporter ($P_{CRP\text{-}D}$). This reporter was inactive in a Δ*crp* mutant or in wild type grown on glucose, but it displayed activity in symbiotic wild-type *V. fischeri* cells (Figure 2.3A). These data indicate that cAMP-CRP is an active regulator in symbiotic cells and suggests the same general relationship between cAMP-CRP activity and glucose that is seen in other bacteria. In *E. coli*, cAMP-CRP levels are lowered in the presence of glucose through the regulation of cAMP levels by the glucose PTS and by lowering the amount of CRP protein (69). *V. fischeri* also has annotated components of the glucose PTS that share homology with the *E. coli* proteins (155). Thus, the relatively low expression of the $P_{CRP\text{-}D}$-*gfp* reporter in glucose-grown *V. fischeri* cells is not surprising. This observation is also consistent with the much-improved relative growth of the Δ*crp* and Δ*cyaA* mutants when they are provided glucose indicating that growth on glucose requires less cAMP-CRP activity. Although there may be many causes for the attenuation of the Δ*crp* and Δ*cyaA*

mutants in the host light organ, the observations with our reporter in wild-type cells support the conclusion that glucose is not a major carbon source for symbiotic cells.

As noted above, there has been speculation on possible carbon sources or growth substrates within the *E. scolopes* light organ. Experiments with auxotrophs suggest the light organ is a complex environment (59), and Wier *et al.* suggested that different substrates are utilized at different times during the symbiosis (164). Of particular interest is NAG, which makes up the polymer chitin and is abundant in the marine environment, constituting an important carbon and nitrogen source for many vibrios such as *V. cholerae* (56). Even in non-marine models, NAG can be an important growth substrate for host-associated bacteria (27, 139). Similar to *V. cholerae* (56), *V. fischeri* has multiple copies of *nag* catabolic genes (103), and there are two putative CRP binding sites within the intergenic region between the divergent *nagE-nagAC* operons in *V. fischeri*, suggesting that cAMP-CRP activates these promoters, as was shown at the similar operon in *E. coli* (120) and *V. cholerae* (56). Although a *nagB* mutant did not have an obvious colonization defect (103), NAG could still be a growth substrate available to symbiotic cells.

In the present study, our data with NAG were somewhat surprising and difficult to interpret. Despite the bioinformatic prediction that cAMP-CRP might be required to stimulate NAG catabolism, NAG recovered growth of Δ*crp* and Δ*cyaA* mutants similarly to glucose (Table 2.2), indicating that it is efficiently catabolized without CRP. Moreover, in contrast to their similar effects on growth, the bright green fluorescence seen with the $P_{CRP\text{-}D}$-*gfp* reporter in wild-type cells grown on NAG was the opposite of the effect seen with glucose. These observations might be rationalized as NAG not being transported by the glucose PTS, and therefore not affecting CyaA activity and cAMP levels, yet still providing the cells with easy access to glucose

through NAG catabolism. However, a growth analysis of a *V. fischeri* $EIIA^{Glc}$ mutant on NAG suggests $EIIA^{Glc}$ may be involved in NAG catabolism (data not shown). Moreover, one might expect the same argument to be true for cellobiose, which is a disaccharide of glucose that has a dedicated transport system (1), yet cellobiose does not recover growth of our mutants as well as NAG did (Table 2.2). The relative efficiency and kinetics of cellobiose and NAG utilization, or the relative dependence on CRP for their catabolism, may account for the differences we have observed. Finally, it is worth noting that others have suggested that metabolic effects of glucose, for example its ability to provision the cell with certain metabolic precursors, affects cAMP-CRP activity rather than glucose per se (171); however, the distinction between NAG and glucose in our study does suggest a special effect of glucose itself.

Although the CRP regulon in *V. fischeri* has not been entirely defined, we have provided insight into its importance in carbon source utilization in culture and symbiotic growth in the light organ. In particular, our data with a fluorescent $P_{CRP\text{-}D}$-*gfp* reporter highlight the activation of cAMP-CRP in symbionts. Future studies with this reporter comparing symbionts over time and in different light-organ microenvironments will help to elucidate the nutritional status of *V. fischeri* symbionts.

Acknowledgements

We thank Alex Horswill for providing pAH9. The National Science Foundation supported this research under grants OCE-0929081 and IOS-1121106.

CHAPTER 3

THE PARADOXICAL EFFECT OF GLUCOSE ON INTRACELLULAR CAMP AND CAMP-CRP ACTIVITY IN *VIBRIO FISCHERI*[2]

[2]Deanna M. Colton and Eric V. Stabb. To be submitted to *Journal of Bacteriology*

Abstract

Proteobacteria often coordinate regulatory responses to carbon sources using CRP and the second messenger cAMP, which combine to stimulate transcription of genes during growth on non-glucose substrates. For example, pheromone signaling in *Vibrio fischeri* is controlled in part by the availability of glucose and this effect relies on cAMP-CRP. CRP typically plays a role in catabolite repression and consistent with that model, a cAMP-CRP-dependent reporter showed lower activity in *V. fischeri* grown in media amended with glucose rather than glycerol. Surprisingly though, intracellular cAMP levels were higher in glucose-grown cells. Using mutant analyses, we established that adenylate cyclase, CyaA, was responsible for cAMP generation, the $EIIA^{Glc}$ component of the glucose transport system could enhance cAMP production, and the phophodiesterases, CpdA and CpdP, consumed intracellular and extracellular cAMP, respectively. However, the observation of lower intracellular cAMP levels in glycerol-grown cells seemed best explained by changes in cAMP export, via an unknown mechanism. Importantly, the cAMP-CRP-dependent reporter had lowered activity in response to glucose even when a *crp2* allele, which acts independently of cAMP, was placed in a Δ*cyaA* mutant background, indicating that the inhibitory effect of glucose was independent of cAMP. Neither *crp* transcription nor CRP protein levels appeared to account for this effect of glucose. We speculate that some unknown mechanism, perhaps differential post-translational modification of CRP during growth on glycerol or glucose, may modulate cAMP-CRP activity.

Introduction

Cyclic 3', 5'-AMP (cAMP) together with the cAMP receptor protein CRP are widespread in Proteobacteria and are best known for their role in activating expression of catabolic pathways in response to growth on a non-preferred (e.g., non-glucose) substrate (58, 100, 121). When cAMP combines with CRP, it causes a conformational change such that CRP binds target promoters, allowing it to interact with RNA polymerase and activate or inhibit transcription (77). As discussed below, cAMP-CRP activity typically is decreased when glucose is available, which plays a role in the catabolite repression response. Accordingly, CRP-mediated regulation encompasses many metabolic pathways and non-metabolic processes as well.

Within the *Vibrionaceae*, CRP plays the traditional role in regulating catabolic genes, but it also controls processes involved in infection and pheromone-mediated signaling. For example, motility, virulence, pheromone signals, and quorum-sensing regulators are controlled by CRP in the human pathogens *Vibrio vulnificus* and *Vibrio cholerae* (75, 85). In the mutualistic light-organ symbiont *Vibrio fischeri*, CRP modulates both the LuxI/LuxR and AinS/AinR pheromone systems, which in turn regulate colonization factors such as motility and luminescence (87, 88, 91). *V. fischeri* is an useful model for understanding regulation during infection, in part because of the experimental tractability of its symbiosis with *Euprymna scolopes* (126, 141). Elsewhere, we have reported that cAMP-CRP is active and important during this symbiotic infection, and we became interested in how cAMP-CRP activity is modulated in *V. fischeri*.

The role of cAMP-CRP in catabolite repression has been elucidated primarily in *Escherichia coli*, although studies in *V. vulnificus* and *V. fischeri* are also useful (35, 37, 75). In *E. coli*, the model has emerged that cAMP-CRP activity is controlled through the modulation of the second messenger cAMP, and that intracellular cAMP levels are controlled at the level of

adenylate cyclase (CyaA), which generates cAMP (168). A connection to glucose availability is provided by the glucose phosphotransferase system $EIIA^{Glc}$ component, the phosphorylated form of which increases CyaA activity (83). When glucose is available, it is phosphorylated after its transport into the cell, with the consequence of less phosphorylated $EIIA^{Glc}$ and therefore less CyaA enzymatic activity and synthesis of cAMP (6, 9, 48, 119, 150).

Other means of cAMP control include cAMP degradation and cAMP export. In *E. coli*, turnover of cAMP is accomplished by the cAMP phosphodiesterase CpdA (67, 73). Interestingly, *V. fischeri* possesses both CpdA and a periplasmic cAMP phosphodiesterase that *E. coli* does not (22, 36). The periplasmic cAMP phosphodiesterase, CpdP, in *V. fischeri* allows it to grow on cAMP as a sole nitrogen and carbon source, but its role, if any, in controlling endogenously produced cAMP is unknown. Bacteria also release cAMP, and it was proposed that export is the main control over cAMP in *E. coli*, given CpdA's relatively low affinity for cAMP (61).

Despite the focus on control of cAMP levels, it remains possible that cAMP-CRP activity is controlled at least in part by other mechanisms. For example, at least under some conditions, CRP protein levels are lower during growth on glucose (69). Moreover, it should be cautioned that some studies use native β-galactosidase activity to assess changes in cAMP levels (47, 61), and this indirect measure might just as well reflect other effects on the CRP protein.

In this study we sought to measure cAMP in *V. fischeri* and to assess the roles of CyaA, CpdA, CpdP, and $EIIA^{Glc}$ in setting these levels. We also wanted to test the overarching predictions, based on other systems, that cAMP-CRP-dependent activation would be higher when cells were grown on glycerol rather than glucose, and that this difference in activity would reflect lower intracellular cAMP levels in glucose-grown cells.

Materials and Methods

Strains, growth media and reagents

Strains, plasmids, and oligonucleotides used in this study are listed in Table 3.1. *V. fischeri* ES114 was the wild-type strain used (12). *E. coli* strains DH5α (60) or DH5αλpir (39) were used for cloning, with the latter used to maintain plasmids with the R6K origin of replication. *E. coli* cultures were grown at 37ºC in LB medium (101) with 20 μg ml^{-1} chloramphenicol (cam) or 40 μg ml^{-1} kanamycin (kan) added when appropriate for selection. *V. fischeri* was grown at 28ºC in LBS medium (144), at 24ºC in SWTO medium (14), or at 24ºC in supplemented mineral salts medium (FMM) (134) containing 40 mM glycerol or 20 mM glucose. When appropriate for selection of *V. fischeri*, 2 μg ml^{-1} cam or 5 μg ml^{-1} erythromycin (erm) were added to LBS.

Plasmids, oligonucleotides, molecular techniques, and sequence analyses

Plasmids and mutants were constructed using standard techniques as previously described (15, 76). Once mutant alleles were constructed, they were transferred from *E. coli* into *V. fischeri* on plasmids by triparental mating using the conjugative helper plasmid pEVS104 (145) in strain CC118λ*pir* (64), and allelic exchange was confirmed by PCR.

For the construction of the Δ*cyaA crp1* strain DC25, the *E. coli crp** allele was amplified from pCRP (CRP* T127L/S128I) using oligos EcCRPfor and EcCRPrev. The *crp** allele was then cloned into pDC41 between sequences flanking *crp* in *V. fischeri*. We generated pDC41 from pJLB117, which contains a Δ*crp* allele with the start and stop codons of *crp* separated by an *Nhe*I site. pJLB117 was digested with *Xba*I and self-ligated to delete a second *Nhe*I site. pBluescript was fused to this vector at a *Xba*I site, because its ColE1 origin facilitates allelic

exchange, and the resulting plasmid was pDC41. The amplified *crp** allele was then cloned into the unique *Nhe*I site between the sequences upstream and downstream of *crp* to make pDC42. pDC42 was then introduced into Δ*cyaA* strain DC03, and through allelic exchange it replaced the native *crp* with *crp** (Table 3.1). As explained further below, sequencing this locus revealed that recombination into the chromosome occurred in a region of shared homology between *E. coli crp** and *V. fischeri crp*, resulting in a *V. fischeri-E.coli crp** hybrid (*crp1*) in strain DC25. A spontaneous mutation in the *crp1* allele resulted in *crp2* in strain DC26.

To create a chromosomal CRP-dependent reporter, we first generated plasmid pDC16, which allows us to place the native *luxCDABEG* luminescence-generating genes under control of a non-native promoter following allelic exchange. Briefly, pDC16 contains *V. fischeri* sequences from within *luxR* to within *luxC*, centered on the intergenic region between *luxI* and *luxC*, with three transcriptional terminators and a multiple cloning site between *luxI* and *luxC* oriented such that promoters cloned into the multiple cloning site will be responsible for *luxCDABEG* expression when exchanged into the chromosome. To create the promoterless-*lux* strain DC08, pDC16 was exchanged into ES114. The artificial CRP-dependent promoter $P_{CRP\text{-}D}$ from pDC85 was cloned upstream of *luxC* in pDC16 to make pDC82. This allele was exchanged into ES114 and DC26 to make strains DC57 and DC63, respectively, so that *luxCDABEG* in these strains is under transcriptional control of an artificial cAMP-CRP-dependent promoter.

To generate a mutant lacking *tolC* (VF2233), we generated a deletion allele consisting of 1.5 kb upstream of *tolC* (amplified with primers tolColigoA and tolColigoB) fused at a *Nhe*I site to 1.5 kb of downstream of *tolC* (amplified with primers tolCdownF2 and tolCdownR2) in plasmid pDC70, which was moved into ES114 or DC18 for allelic exchange, resulting in Δ*tolC* and Δ*cpdP* Δ*tolC* mutants DC52 and DC53, respectively (Table 3.1).

To generate *crr*::*ermR* mutants by single crossover and plasmid integration, plasmid pTMO152 was moved into the recipient *V. fischeri* strains through triparental mating. To make *crr*::*erm* mutants DC42 and DC51, pTMO152 was moved into DC38 and DC18, respectively. To make the Δ*crp* P_{CRP-D}-*lux* strain, DC68, pJLB117 was moved into DC57 and through allelic exchange, the *crp* gene was knocked out.

Luminescence measurements

Overnight *V. fischeri* cultures were diluted 1:1000 in 20 ml of SWTO or FMM in 125-ml flasks, and then incubated at 24˚C with shaking at 200 rpm. 500-µl samples were removed periodically and the optical density at 595 nm (OD_{595}) was measured with a BioPhotometer (Brinkman Instruments, Westbury, NY). Samples were aerated by rapid shaking for 10 seconds, and relative luminescence was measured immediately with a Glomax TD-20/20 luminometer (Promega, Madison, WI) (15). Specific luminescence reported is the relative luminescence per OD_{595}.

cAMP Assays

We used the Amersham cAMP Biotrak Enzyme-immunoassay System (GE Healthcare, Buckinghamshire, UK) to determine cAMP concentrations following the manufacturer's instructions. For measurement of intracellular cAMP, cells cultured in FMM containing either glycerol or glucose were grown to an OD_{595} ~0.4, and 5 ml of culture were pelleted by centrifugation. The pellet was washed twice with cold FMM, re-suspended in 270 µl assay buffer, and boiled for 10 min. Then 30 µl of 2.5% dodecyltrimethylammonium bromide (lysis 1A reagent) was added to give a final concentration of 0.25%. The lysate was pelleted by

centrifugation to remove debris, and the supernatant was either stored at -80ºC overnight or loaded directly into the assay wells. The manufacturer's instructions were then followed to assay cAMP levels. For detection of extracellular cAMP, 1 ml of culture was sampled, cells were removed by centrifugation, the supernatant was diluted with lysis 1A reagent, and 100 µl of the samples were loaded into wells. For each experiment, two biological replicates were run for each condition in duplicate wells. For measurements of extracellular cAMP, the cAMP standard was prepared in FMM + 0.25% lysis solution to match the samples. The absorbance at 450 nm was measured using a Synergy 2 plate reader (BioTek, Winooski, VT), and the concentrations of cAMP in the samples were determined from the standard curve and normalized to the protein concentration of the lysate measured by a Bradford Assay using Protein Assay reagent from Biorad (Hercules, CA).

Another kit, DetectX Direct cyclic AMP Enzyme Immunoassay (Arbor Assays, Ann Arbor, Michigan) was used to assay intracellular and extracellular cAMP for DC48. For detection of intracellular cAMP, cells were grown as above except that 10 ml of cells were pelleted by centrifugation and lysed with 200 µl of the supplied sample diluent. Extracellular cAMP samples and standards were prepared as above.

Western blot analysis of CRP

V. fischeri strains grown in glycerol or glucose were lysed by boiling in SDS reducing buffer (65 mM Tris-HCl with 2% SDS) and pelleted by centrifugation. The soluble proteins were resolved using SDS-PAGE and transferred to a polyvinyldene fluoride (PVDF) membrane (Millipore, Bedford, MA). *E. coli* strain MG1655 was included as a positive control and *V. fischeri* JB24 Δ*crp* mutant was used as a negative control. The total protein was visualized by

staining with Ponceau S (0.1% Ponceau S in 1% acetic acid) and de-stained with 0.1 M sodium hydroxide and distilled water. The membrane was blocked with 5% nonfat dry milk overnight at 4°C with shaking. After extensive washing with Tris-buffered saline and Tween 20 (TBS-T) (0.02 M Tris-HCl pH 7.6; 16 g l^{-1} NaCl; 0.2% Tween 20), the membrane was probed with a 1:2000 dilution of polyclonal rabbit anti-*E. coli*-CRP antibody (Hiroji Aiba group, Japan) as the primary antibody and washed again with TBS-T and then incubated with a 1:2000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Biorad, Hercules, CA) as the secondary antibody. The secondary antibody was detected using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) according to the manufacturer's instructions (Promega, Madison, WI).

Table 3.1: Bacterial strains and plasmids used in this study

Strain and plasmid	Relevant characteristics[a]	Source or reference
E. coli		
MG1655	F- lambda- *ilvG- rfb*-50 *rph*-1	(10)
DH5α	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(60)
DH5α*λpir*	DH5α lysogenized with *λpir*	(39)
CC118*λpir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	(145)
V. fischeri		
DC03	ES114 Δ*cyaA*	Chpt. 2
DC08	ES114 promoterless-*lux*	This study
DC18	ES114 Δ*cpdP*	Chpt. 2
DC25[b]	ES114 Δ*cyaA crp1*	This study

DC26[c]	ES114 Δ*cyaA crp2*	This study
DC38	ES114 Δ*cpdA*	Chpt. 2
DC42	ES114 Δ*cpdA crr::ermR*	This study
DC48	ES114 Δ*cpdP* Δ*cpdA*	Chpt. 2
DC51	ES114 Δ*cpdP crr::ermR*	This study
DC52	ES114 Δ*tolC*	This study
DC53	ES114 Δ*cpdP* Δ*tolC*	This study
DC57	ES114 P_{CRP-D}-*lux*	This study
DC63	ES114 Δ*cyaA crp2* P_{CRP-D}-*lux*	This study
DC68	ES114 Δ*crp* P_{CRP-D}-*lux*	This study
ES114	Wild-type isolate from *E. scolopes*	(12)
JB22	ES114 $P_{AI/34}$-*luxCDABEG*	(15)
JB24	ES114 Δ*crp*	(14)
JAS202	ES114 Δ*crr*	J. Schwartzman
Plasmids[d]		
pAKD702	Promoterless-*lacZ*, pES213, *oriV*R6Kγ, $oriT_{RP4}$, *camR*	(42)
pBluescript	ColEI, *ampR*	Stratagene
pCRP	*E. coli crp** (CRP* T127L/S128I)	(172)
pDC16	*luxR-luxI*; three transcriptional terminators; promoterless-*luxC*	This study
pDC41	Δ*crp* allele Δ*Nhe*I; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *camR*	This study
pDC42	*E. coli crp** allele; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *camR*, *ampR*	This study
pDC70	Δ*tolC* allele; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *camR*	This study
pDC82	*luxR-luxI*; transcriptional terminators; P_{CRP-D}-*luxC*	This study
pDC85	*oriV*R6Kγ, $oriT_{RP4,}$ pES213, *mCherry*, *kanR*, P_{CRP-D}-*camR-gfp*	Chpt. 2
pEVS104	conjugative helper plasmid; *oriV*R6Kγ, $oriT_{RP4}$, *kanR*	(145)
pJLB117	Δ*crp* allele; ColE1, *oriV*R6Kγ, $oriT_{RP4}$, *kanR*, *camR*	(14)

pJLB146	P*lac-crp* in shuttle vector pVSV105; pES213, *oriV*R6Kγ, *camR*	(14)
pTMO152	*crr*::*ermR*; *oriV*R6Kγ	(155)
Oligonucleotide[e]		
Pcrp92#1	CTA ATG TGA GTT AGC TCA CTC ATA GTT TAT CAA AAA ATA CAC TAC AAT ACT GTC GAC	This study
Pcrp92#2	CTA GGT CGA CAG TAT TGT AGT GTA TTT TTT GAT AAA CTA TGA GTG AGC TAA CTC ACA TTA GGG CC	This study
EcCRPfor	CGA ACG ACT AGT GCA TGG TGC TTG GCA AAC CGC	This study
EcCRPrev	CGA ACG ACT AGT GGG ATT AAC GAG TGC CGT AAA CGA CGA TGG	This study
tolColigoA	CAA CGG TGT GAT CTT GAG AAA TAT CAC CCG	This study
tolColigoB	GCT AGC CTC ATG GTG GTT TGT CCT GC	This study
tolCdownF2	GTA GCT AGC CCA AAT AAG TTG ATA CCA ATT ATG TAA TTA C	This study
tolCdownR2	CGA CTA AGT TGG CGT AGA ACT TGC G	This study

[a] Drug resistance abbreviations used: *camR*, chloramphenicol resistance; *ermR*, erythromycin resistance; *kanR*, kanamycin resistance; *ampR*, ampicillin resistance

[b] Upon recombination into *V. fischeri* chromosome, *E. coli crp** became *crp1* (*V. fischeri-E. coli crp** hybrid)

[c] A spontaneous mutation occurred to make additional amino acid change (G141S) in *crp1* to make *crp2*

[d] Replication origin(s) of each vector are listed as R6Kγ, ColE1, and/or pES213. Plasmids based on pES213 are stable and do not require antibiotic selection for maintenance (40).

[e] All oligonucleotides are shown 5' to 3'. Underlined regions highlight restriction enzyme recognition sites.

Results

Intracellular cAMP levels are higher in cells grown on glucose

Given the role of cAMP-CRP in the catabolite-repression response of *E. coli* and other Proteobacteria (17, 34, 121), along with the observation that *cyaA* and *crp* are more dispensable in *V. fischeri* when cells are provided glucose (91), we predicted that *V. fischeri* cells growing on glycerol would have higher intracellular cAMP levels than cells growing on glucose. However, we observed the opposite, with ~3-fold higher levels of intracellular cAMP in wild-type ES114 cells grown on glucose relative to glycerol (Fig. 3.1). This result was reproducible and statistically significant ($p<0.05$). We estimate that the intracellular cAMP concentrations in ES114 grown under these conditions were approximately 15 to 45 µM, which falls within a

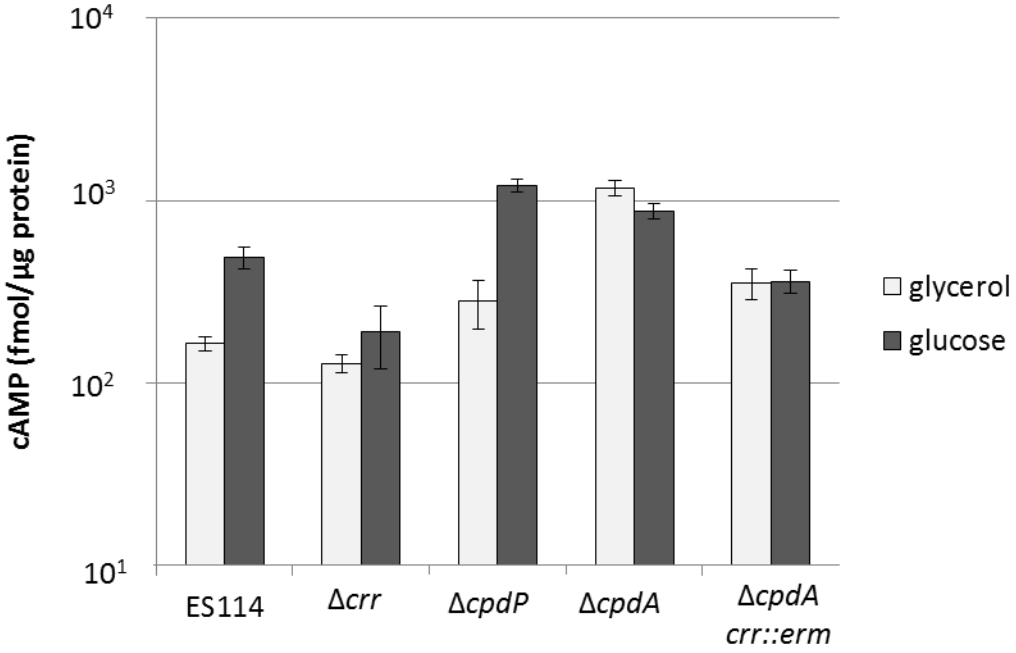

Figure 3.1: Intracellular cAMP for *V. fischeri* strains. Cells were grown to OD_{595} of 0.4 in FMM amended with 40 mM glycerol or 20 mM glucose. cAMP in cell lysate was normalized to µg of protein. Error bars represent standard error. Data from multiple experiments were combined with ES114 in each experiment giving similar values. We estimate that 100 fmol cAMP/µg protein on this scale is equivalent to an intracellular concentration of ~10 µM cAMP.

range that would be physiologically relevant in *E. coli* (116, 118). Figure 3.1 reports cAMP levels for cells at 0.4 OD_{595}; however, we similarly observed higher cAMP levels in glucose-supplemented cells at OD_{595} of 0.2 and 0.6 (data not shown).

Control of intracellular cAMP levels by CyaA, CpdA, and $EIIA^{Glc}$

We sought to test the roles of proteins that might influence cAMP levels in the cell, and if possible to find a mechanism underlying the higher intracellular levels of cAMP observed in glucose-grown cells. Based on other Proteobacteria and bioinformatic analysis of the ES114 genome we predicted that: (i) CyaA is responsible for cAMP synthesis, (ii) intracellular cAMP is consumed by the phosphodiesterase CpdA, (iii) cAMP levels may be modulated further by the periplasmic phosphodiesterase CpdP, and (iv) cAMP production is enhanced by $EIIA^{Glc}$ (encoded by *crr*) in the absence of glucose. To test this model, we generated mutants in *cyaA*, *cpdA*, *cpdP*, and *crr*, both singly and in specific combinations, and measured intracellular cAMP levels.

CyaA was the only putative adenylate cyclase identified in the *V. fischeri* ES114 genome, and a Δ*cyaA* mutant produced no detectable cAMP. This strain served as a negative control as measurements of its cAMP were consistently at background levels, which were typically less than 1% of the intracellular cAMP concentration of ES114 grown on glycerol (data not shown).

The ES114 genome revealed two putative cAMP phosphodiesterases, CpdA and CpdP. The latter had previously been characterized in *V. fischeri* as a periplasmic cAMP phosphodiesterase (22, 36), and we found that deleting *cpdP* resulted in 1- to 2-fold higher intracellular cAMP compared to ES114 (Fig. 3.1). Moreover, the Δ*cpdP* mutant still displayed

~3-fold higher intracellular cAMP when grown on glucose, much like wild type (Fig. 3.1). A more likely candidate for influencing intracellular cAMP levels appeared to be CpdA, which resembled cytoplasmic cAMP phosphodiesterases from *E. coli* (67) and *V. vulnificus* (73), both in its sequence and the genetic context of the *cpdA* gene. Indeed, the Δ*cpdA* mutant had significantly elevated intracellular cAMP levels relative to ES114 ($p<0.05$), particularly when grown on glycerol (Fig. 3.1), confirming its role in breaking down intracellular cAMP. Unlike ES114 and the Δ*cpdP* mutant, for the Δ*cpdA* mutant there was relatively little difference between glucose- and glycerol-grown cells (Fig. 3.1).

Next, to test the role of $EIIA^{Glc}$ in enhancing cAMP production, we measured cAMP in a Δ*crr* mutant and moved this mutation into the Δ*cpdA* and Δ*cpdP* backgrounds. Our primary prediction was that a Δ*crr* mutant would have lower cAMP levels than wild type when grown on glycerol, owing to a lack of phosphorylated $EIIA^{Glc}$, which is thought to stimulate CyaA activity

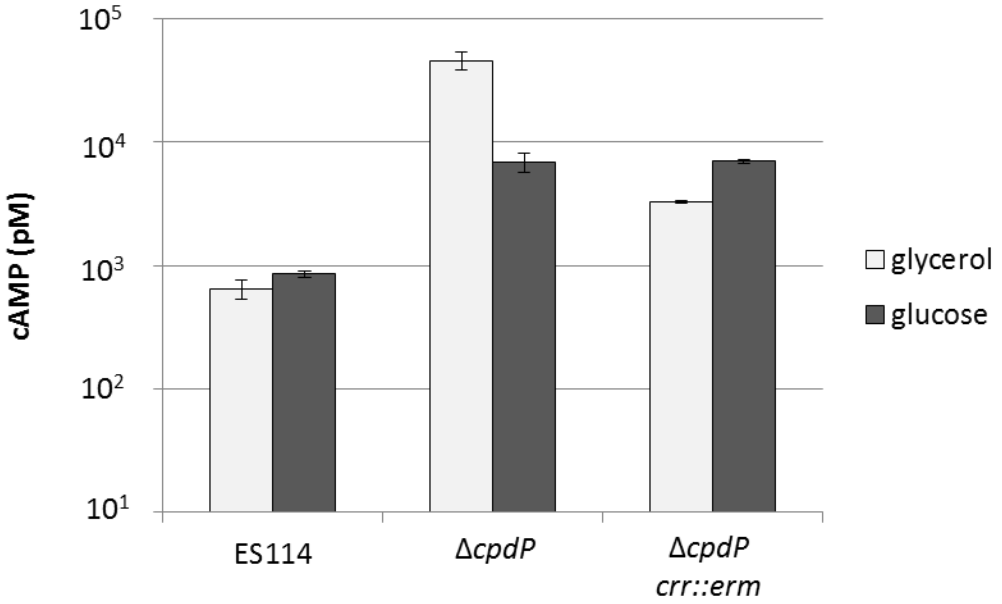

Figure 3.2: Extracellular cAMP in *V. fischeri* cultures. Strains were grown to OD_{595} of 0.4 in FMM amended with 40 mM glycerol or 20 mM glucose. Error bars represent standard error. Data from multiple experiments were combined with ES114 in each experiment giving similar values.

in *E. coli* (6, 48, 100, 119, 121, 130, 150). We saw no significant difference from ES114 ($p>0.05$) in cAMP levels in the Δ*crr* mutant in glycerol-grown cells; however, when a Δ*cpdA* mutant was compared to a Δ*cpdA crr*- double mutant, a significant ($p<0.05$) drop in intracellular cAMP was seen (Fig. 3.1). Although *crr* mutants grew poorly relative to other strains on glucose, *crr* mutants did achieve the 0.4 OD_{595} at which cAMP levels were measured.

Extracellular cAMP differs in glucose- and glycerol-grown cells

cAMP export has been reported in other bacteria (131), and in *E. coli* extracellular cAMP can constitute ~90% of the total cAMP in culture (61, 118) We therefore considered the possibility that differential cAMP export under various growth conditions could account for changes in intracellular cAMP. We measured extracellular cAMP in wild type grown on glycerol or glucose and saw no difference ($p>0.05$) in extracellular cAMP (Fig. 3.2). However, the periplasmic cAMP phosphodiesterase CpdP might be expected to consume extracellular cAMP and obscure any differences in cAMP export. In the Δ*cpdP* mutant we observed 10- to 100-fold more extracellular cAMP than wild-type ES114 grown on glucose or glycerol, respectively (Fig 3.2). Additionally, extracellular cAMP was 7-fold higher in glycerol-grown cells as opposed to glucose (Fig. 3.2). Moreover, during growth on glucose, a Δ*cpdP crr*- double mutant had over 10-fold less extracellular cAMP than did the Δ*cpdP* mutant. These results are consistent with a model whereby $EIIA^{Glc}$ contributes to increased cAMP production during growth on glycerol, but that concomitantly increased export of cAMP in glycerol-grown cells results in lower intracellular cAMP levels.

To test the importance of cAMP export further, we measured intracellular and extracellular cAMP in the same cultures using a Δ*cpdP* Δ*cpdA* mutant, allowing us to assess total

cAMP export without interference from cAMP degradation. We found that in glycerol-grown cells the combined intracellular and extracellular cAMP was actually 2-fold higher than in glucose-grown cells (Fig. 3.3). However, only 24% of the total cAMP in cultures of glycerol-grown cells is intracellular, whereas 76% of total cAMP remained intracellular in glucose-grown cells at the same OD_{595}.

Hantke *et al.* postulated that the outer membrane channel protein TolC was involved in cAMP export in *E. coli* (61), and we therefore investigated TolC in *V. fischeri*. However, we did not see an accumulation of intracellular cAMP in a Δ*tolC* mutant, nor did we find a difference in extracellular cAMP levels between a Δ*tolC* mutant and a Δ*cpdP* Δ*tolC* double mutant (data not shown). We then employed a random transposon mutagenesis approach (93) in an attempt to find mutants defective in cAMP export. We used the Δ*cpdP* Δ*cpdA* Δ*cyaA* triple mutant DC55, which can neither generate nor destroy cAMP, as the parent strain, and monitored its ability to

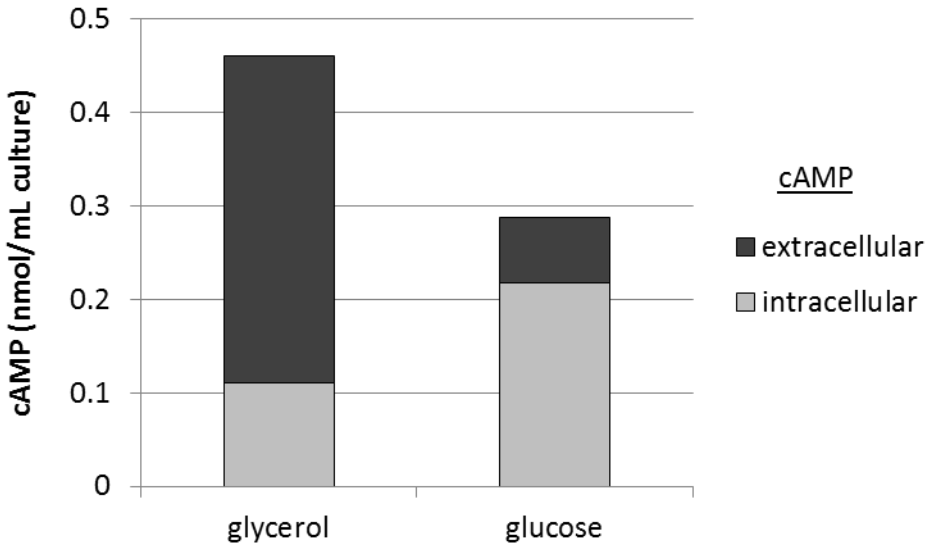

Figure 3.3: Comparison of intracellular cAMP to extracellular cAMP. Both intracellular and extracellular cAMP was measured from cultures of DC48 (Δ*cpdP* Δ*cpdA*) grown in FMM amended with 40 mM glycerol or 20 mM glucose to an OD_{595} of 0.4.

utilize mannose using a colorimetric assay. Exogenous addition of sufficient cAMP could stimulate mannose utilization in DC55, and we screened mutants for mannose utilization in the presence of sub-stimulatory cAMP levels reasoning that impairment of cAMP export might allow mannose utilization to be stimulated with lower exogenous cAMP levels. Unfortunately, after screening several thousand mutants, none were obtained with this phenotype.

cAMP-CRP activity appears lower in cells grown on glucose

Given our unexpected observation that intracellular cAMP levels were actually higher in glucose-grown cells, we wanted to determine how glucose affected cAMP-CRP activity. In

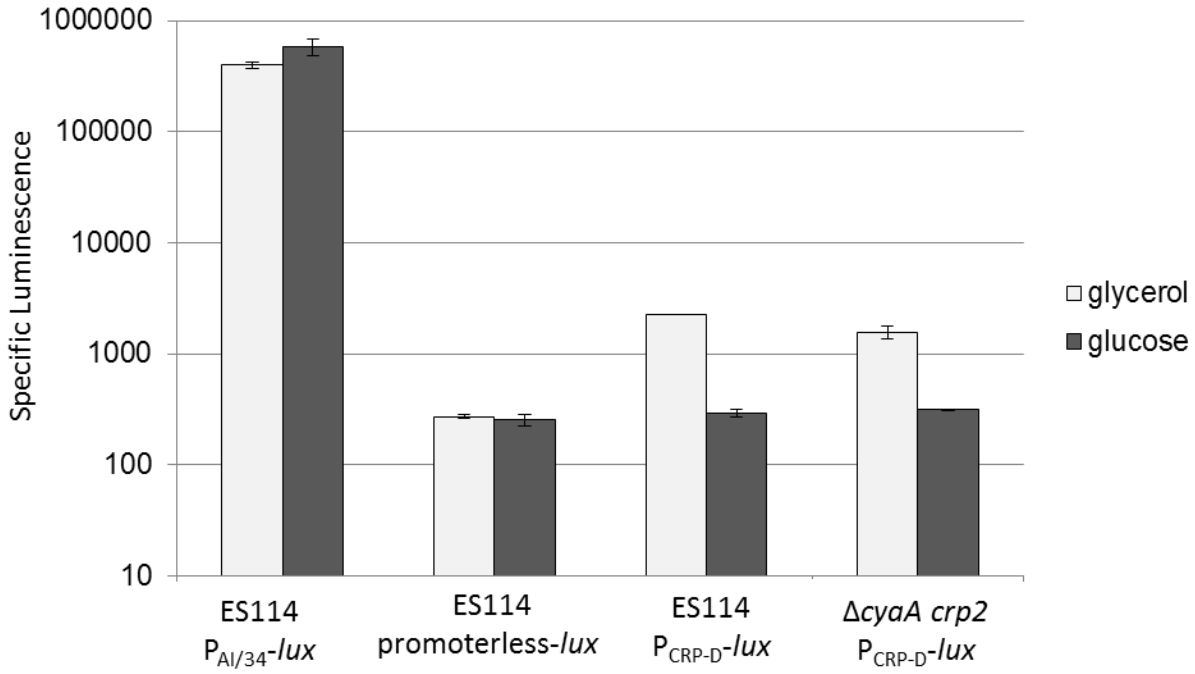

Figure 3.4: Expression of CRP-dependent reporter in *crp*$^+$ and *crp2*$^+$ backgrounds. Specific luminescence was determined for cells grown to OD_{595} of 1.0 on SWTO amended with 40 mM glycerol or 20 mM glucose. Background luminescence is represented by an ES114 derivative with a promoterless-*lux* allele. The ES114 strain with constitutive-*lux* serves as control to show the glucose effect on reporter activity is CRP-dependent. Error bars represent standard error (n=2).

other cases, intracellular cAMP levels dictate cAMP-CRP activity; however, to fill its traditional role in catabolite repression cAMP-CRP activity would have to be lower on glucose-grown cells. To test cAMP-CRP activity in transcriptional activation, we synthesized an artificial cAMP-CRP-dependent promoter ($P_{CRP\text{-}D}$) and engineered a strain where the *luxCDABEG* genes were placed under $P_{CRP\text{-}D}$ control on the *V. fischeri* chromosome. Consistent with the traditional role of CRP in catabolite repression, we saw ~10-fold lower activity from the CRP-dependent reporter in cells grown on glucose relative to those grown on glycerol (Fig. 3.4). Similar results were obtained with a plasmid-borne $P_{CRP\text{-}D}$-*gfp* reporter (data not shown). Additionally, JB22 (ES114 $P_{AI/34}$-*lux*), a constitutive *lux*-expressing strain grown on glycerol or glucose, was used as a control to show that the glucose effect on luminescence in the reporter strains was dependent on CRP (Fig. 3.4).

The effect of glucose on cAMP-CRP activity is independent of cAMP

Given our apparently paradoxical observations that amendment with glucose appears to simultaneously decrease cAMP-CRP activity while increasing intracellular cAMP, we wanted to determine if the effect of glucose on cAMP-CRP activity was independent of cAMP levels. To accomplish this goal, we generated a strain with a *crp* that acts independently of cAMP, *crp**, in a Δ*cyaA* mutant background. We obtained an *E. coli* cAMP-independent *crp** allele with amino acid changes T127L/S128I (172) that we exchanged onto the chromosome of a Δ*cyaA* mutant at the *crp* locus. Unexpectedly, recombination occurred between the *E. coli crp** sequence and the *V. fischeri crp* gene, resulting in a chimeric *crp* gene that encoded the first 63 amino acids of *V. fischeri* CRP, and the remaining C-terminal *E. coli crp* sequence, including the mutations encoding the T127L/S128I CRP* changes. We noticed two colony phenotypes when streak-

purifying this strain; one that was slightly smaller than wild type and one closer to wild-type size. Both types were isolated and *crp** from each was sequenced. A clone with the large-colony phenotype had an additional mutation in *crp*, generating amino acid change G141S. A similar G141S mutation was documented previously by Kim *et al.* to make constitutively active CRP variants (74). We used this T127L/S128I/G141S CRP* variant (*crp2*) in a Δ*cyaA* background, so that cells lacked cAMP yet displayed phenotypes consistent with activation of the CRP regulon.

Interestingly, glucose still caused decreased expression of the $P_{CRP\text{-}D}$-*lux* reporter, even in Δ*cyaA crp*2 background (Fig. 3.4). We previously reported that under these conditions, glucose did not affect luminescence from a similarly constructed strain with a constitutive promoter driving *lux* expression (91). Thus, under these conditions, the glucose effect on CRP activity appears to be independent of cAMP.

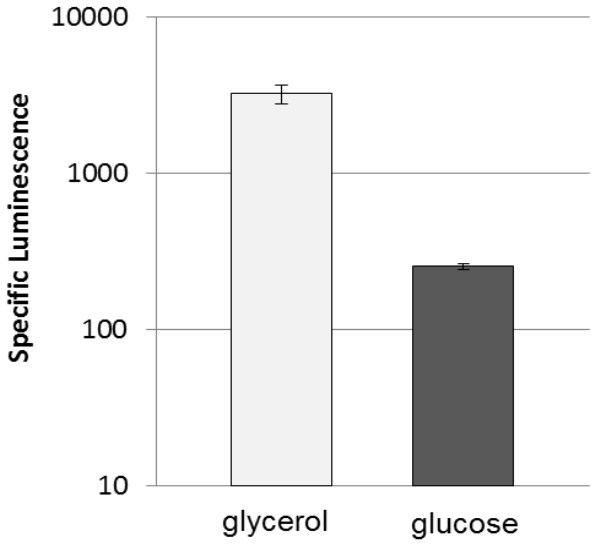

Figure 3.5: Expression of CRP-dependent reporter with constitutive *crp.* Specific luminescence was determined for DC68 (Δ*crp* $P_{CRP\text{-}D}$-*lux*) with plasmid P_{lac}-*crp* grown to OD_{595} of 1.0 on SWTO amended with 40 mM glycerol or 20 mM glucose. Error bars represent standard error (n=2).

The effect of glucose on cAMP-CRP activity is independent of CRP transcription

To explore whether the native promoter of *crp* is necessary for this glucose-mediated effect, *V. fischeri crp* was cloned downstream of a P_{lac} promoter and moved into a Δ*crp* mutant of *V. fischeri* with $P_{CRP\text{-}D}$-*lux* reporter on the chromosome. After removing native transcriptional control of *crp*, we still saw lower cAMP-CRP-dependent activity in the presence of glucose (Fig. 3.5). These data were similar to the 10-fold difference we saw between glycerol- and glucose-grown cells with the $P_{CRP\text{-}D}$-*lux* reporter strains with *crp* on the chromosome (Fig. 3.4). Thus, this glucose-mediated effect on cAMP-CRP activity is not dependent on native *crp* transcription.

Glucose does not detectably affect the amount of CRP protein

Next, to investigate one other potential means of regulating cAMP-CRP activity, we sought to determine if there were lower levels of CRP protein in glucose-grown cells. Ishizuka *et al.* (69) described a reduction in CRP levels in *E. coli* grown on glucose, and we wanted to see if the amount of CRP protein in *V. fischeri* could account for the difference in CRP activity in cells grown on glycerol or glucose in wild-type ES114. Using an anti-CRP antibody, there appears to be no detectable difference in the amount of CRP protein in *V. fischeri* cells grown on glycerol or glucose (Fig. 3.6). Although we have not quantified [CRP] and cannot rule out a difference when cells are grown with glycerol or glucose, our results do not reflect the sort of changes in CRP reported by Ishizuka *et al.* Thus, CRP protein levels do not appear to account for the paradoxical observation of increased cAMP levels and decreased CRP activity in the presence of glucose.

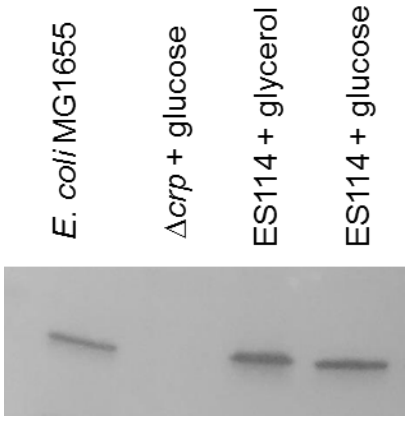

Figure 3.6: Detection of CRP in *E. coli* and *V. fischeri*. Western immunoblot analysis using anti-CRP on cell lysates from *E. coli* MG1655, *V. fischeri* Δ*crp,* and wild-type ES114. *E. coli* grown in LB and *V. fischeri* cells grown to OD_{595} of 1.0 in SWTO amended with 40 mM glycerol or 20 mM glucose.

Discussion

In this study we investigated if and how CRP activity and cAMP levels are affected in response to carbon sources, glucose in particular, in *V. fischeri*. Several lines of evidence suggested that CRP played a role in *V. fischeri* similar to the model that has emerged in other Proteobacteria, with increased activation of the cAMP-CRP regulon when glucose is unavailable. As predicted by this model, Δ*crp* and Δ*cya* mutants of *V. fischeri* grew much better when supplemented with glucose than with glycerol (Chapter 2). Similarly, a Virtual Footprint analysis (108) of the CRP regulon in *V. fischeri* suggested potential CRP binding sites upstream from many genes and operons involved in catabolism of non-glucose carbon sources, such as is the case in *E. coli* and other bacteria. In some instances, experiments seem to confirm these bioinformatic predictions, for example based on the inability of a Δ*crp* mutant to utilize the respective non-glucose carbon sources. Similarly, there is a predicted CRP binding site upstream

from the cellobiose (*cel*) utilization operon, and glucose leads to decreased expression from the *cel* promoter (1). In a recent study (Chapter 2) and here, we generated CRP-dependent reporters, which yielded lower expression when cells were grown in the presence glucose. Taken together, all of our evidence is consistent with the canonical role of CRP, together with the second messenger cAMP, in stimulating expression of certain genes when glucose is unavailable.

Our data are also consistent with established controls over cAMP levels, although the relative importance of these mechanisms appears to be different in *V. fischeri* and *E. coli*, at least under the conditions tested. It appears that in both organisms, the adenylate cyclase CyaA is responsible for cAMP synthesis, and turnover of cAMP is accomplished by the phosphodiesterase CpdA. However, during growth without glucose, an *E. coli cpdA* mutant showed only a 2-fold increase in intracellular cAMP, attributed to a low K_m for cAMP (67), whereas a *V. fischeri* Δ*cpdA* mutant had 10-fold higher cAMP levels (Fig. 3.1). Moreover, as discussed below, the effect of the $EIIA^{Glc}$ transport component on cAMP levels, which appears to be critical in *E. coli* (83), was only evident in *V. fischeri* in a Δ*cpdA* mutant background. Thus it appears that CpdA may play a larger role in determining cAMP levels in *V. fischeri*, and it would be interesting to compare the enzymatic activities of the *V. fischeri* enzyme to that of *E. coli*.

Previous work showed that cAMP levels in *E. coli* are modulated in response to glucose based on the $EIIA^{Glc}$ component of the glucose-transport system, which is encoded by *crr* (132). When glucose is absent, the phosphorylated form of $EIIA^{Glc}$ activates CyaA to produce more cAMP, but in the presence of glucose, $EIIA^{Glc}$ transfers its phosphate to glucose during import, resulting in lower CyaA activity and less cAMP. Bettenbrock *et al.* showed that the phosphorylation state of $EIIA^{Glc}$ dictates the amount of cAMP synthesis (9). Unexpectedly, we

found no significant difference in intracellular cAMP between the Δ*crr* mutant and wild type in glycerol-grown cells; however, the predicted decrease in cAMP upon disruption of *crr* was seen in the Δ*cpdA* mutant and Δ*cpdP* mutant backgrounds in which cAMP levels were elevated (Figs 3.1 and 3.2). These results suggest that $EIIA^{Glc}$ functions similarly in the regulation of cAMP synthesis in *V. fischeri* and *E. coli*, but the relative importance of its role may vary between the organisms or the growth conditions.

A more surprising result was our observation in *V. fischeri* of higher intracellular cAMP levels when cells were grown in the presence of glucose rather than glycerol. This is the opposite of what has been reported in *E. coli* and if cAMP were the determining factor for cAMP-CRP activity, increased cAMP in glucose-grown cells would invert the proposed role of CRP. The higher levels of intracellular cAMP could be in part an effect of differential degradation of cAMP, considering intracellular cAMP was not significantly different in the Δ*cpdA* mutant grown on glycerol or glucose (Fig. 3.1). However, we speculate that cAMP export is the major determinant underlying this difference in intracellular cAMP. Indeed, the relationship between glucose and cAMP was reversed when extracellular cAMP was examined, with higher extracellular cAMP levels from glycerol-grown cells. Upon examining the intracellular to extracellular cAMP concentrations of a Δ*cpdA* Δ*cpdP* double mutant, which eliminates those mechanisms of cAMP turnover, we found that total cAMP is 2-fold higher with glycerol than with glucose (Fig. 3.3). Thus, our data are consistent with higher cAMP production in cells grown in glycerol rather than glucose, but with increased cAMP export in the glycerol-grown cells.

Our results raise intriguing questions about cAMP export. The mechanism underlying this phenomenon is unknown, but does not require *tolC*, as has been proposed in *E. coli* (61). In

E. coli, addition of a *tolC* mutation in a Δ*cya* background increased the sensitivity of a regulatory response to added cAMP, but neither intracellular nor extracellular cAMP was measured directly (61). Given our data, such examination of the *E. coli tolC* mutant seems prudent. Although our initial screen for export-deficient mutants was not fruitful, other approaches might identify the gene(s) involved in cAMP export, which is likely an important research priority for elucidating the role of this second messenger.

It is worth noting that differences in extracellular cAMP concentration were only evident in *V. fischeri* mutants lacking the CpdP periplasmic cAMP phosphodiesterase. CpdP is unlike other previously characterized bacterial periplasmic phosphodiesterases in its specificity for 3'-5'-cyclic nucleotides (22), and our data raise new questions about its role. Upon discovering CpdP, Dunlap *et al.* speculated that it may have a role in degrading free cAMP in the seawater or during symbiotic infection (22). In light of our findings, the selective advantage of CpdP could instead stem from its degradation of endogenously produced cAMP. It is possible that the advantage conferred by CpdP is to recycle exported cAMP by converting it into 5'-AMP, which is subsequently transported back into the cell and funneled into nucleotide synthesis pathways. Alternatively, if host cells respond to cAMP from the symbionts, CpdP might function by attenuating such inter-organismal detection or signaling. Future studies of *cpdP* should consider possible roles related to degradation of endogenous cAMP production.

Perhaps the most important focus for future studies is to understand how glucose affects cAMP-CRP activity under the conditions of our assays, and how such activity could be higher even if cAMP levels are lower. It is not clear why this phenomenon would exist in *V. fischeri* when *E. coli* and other Proteobacteria seem to achieve higher cAMP-CRP activity by increasing intracellular cAMP in response to non-glucose carbon sources. It seems clear that changing

levels of cAMP is only one of multiple ways to modulate cAMP-CRP activity, and the overriding mechanism may depend both on the bacterium and the conditions. It remains possible that the phenomenon we observed in *V. fischeri* also occurs in *E. coli* under certain conditions, and it would be interesting to know if this were the case.

Although we do not know how widespread this phenomenon is, under the conditions used here, there is clearly a glucose-mediated effect independent of cAMP levels. This conclusion is best illustrated by the phenotype of a Δ*cyaA crp2* mutant, which still responded to glucose although it does not generate cAMP and its CRP does not require it. Although others have noted lower CRP protein levels in *E. coli* during growth on glucose (69), we did not see a similar effect to what they reported (Fig. 3.6). Moreover, native transcriptional regulation of *crp* did not appear to be required for the effect of glucose on cAMP-CRP activity.

We speculate that cAMP-CRP activity might be controlled further by post-translational protein modification. One possible mechanism for such control would be lysine acetylation. Alan Wolfe's lab has shown that CRP can be acetylated by acetyl-P *in vitro* in the absence of an acetyltransferase (A. Wolfe, personal communication); however, it is unclear if this process is physiologically relevant. In *Salmonella*, protein acetylation and deacetylation are important mechanisms in regulating many central metabolism enzymes, and acetylation levels can vary between cells grown on glucose or other carbon sources (160). Interestingly, Castano-Cerezo *et al.* provided evidence that cAMP-CRP may regulate the transcription of an *E. coli* acetyltransferase, YfiQ (or PatZ), which is involved in acetate metabolism regulation (25). We considered the possibility that CRP acetylation by YfiQ might underlie the modulation of its activity in glucose-grown cells; however, the same glucose-dependent effect was still seen in a *yfiQ* mutant (data not shown). To investigate further whether CRP is controlled post-

translationally, a good first step would be proteomic analysis on *V. fischeri*'s CRP to identify acetylation or other modifications of this protein in cells grown on glycerol or glucose.

Evidence presented here adds more complexity to the control of cAMP-CRP-mediated gene regulation and provides evidence that carbon sources regulate cAMP-CRP activity in *V. fischeri*, thus proving the importance of an environmental cue in governing pheromone-mediated regulation in the *Vibrio*-squid symbiosis.

Acknowledgements

We thank Ned Ruby and Julia Schwartzman for providing JAS202, Hiroji Aiba for the *E. coli* anti-CRP, and Jorge Escalante-Semerena and Tim Hoover for helpful discussion. The National Science Foundation supported this research under grants OCE-0929081 and IOS-1121106.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this dissertation was to broaden our understanding of how a host environment influences the pheromone-mediated behaviors of a bacterial symbiont. The *Vibrio fischeri-Euprymna scolopes* symbiosis is a great model system for such studies, as bioluminescence is a well-studied pheromone-controlled behavior. *V. fischeri*'s luminescence is much more strongly induced in the host environment than it is in culture, and studying regulators that govern pheromone-mediated regulation can therefore provide insight into the symbiotic environment and help identify the potential cues that underlie the host-symbiont relationship. The regulator CRP, along with the second messenger cAMP, govern pheromone-mediated regulation and bioluminescence in *V. fischeri*, in addition to being involved in other processes, most notably the catabolism of non-glucose carbon sources. My goals were to determine the relevance of cAMP-CRP activity during symbiosis and to examine the mechanisms that regulate this activity in *V. fischeri* grown on different carbon sources.

In Chapter 2, I investigated the importance of cAMP-CRP in the *Vibrio*-squid symbiosis and found that this duo was essential for robust colonization. This observation cannot be explained solely by the dim luminescence of the Δ*cyaA* and Δ*crp* mutants or the known effect of cAMP-CRP on *luxR* and *ainSR*, because a Δ*cyaA* or Δ*crp* mutant is compromised for colonization far more than either a dark mutant (15) or *luxR* and *ainS* mutants (87, 154). It is likely that the severe 100-fold or greater attenuation in colonization by the Δ*cyaA* and Δ*crp*

mutants have multiple causes. cAMP-CRP is known to regulate many colonization factors in other bacteria, for example it regulates type I fimbriation in uropathogenic *Escherichia coli,* which is required for tissue attachment (107), and in *Vibrio cholerae* CyaA and CRP are needed for chitin-induced natural competence, due to cAMP-CRP's role in chitin surface colonization and chitin degradation (11). In *V. cholerae*, cAMP-CRP is involved in regulating HapR, which negatively regulates biofilm production (85). Therefore, *crp* mutants of *V. cholerae* actually have increased biofilm production in addition to cholera toxin production, yet they are unable to colonize the small intestine (84). This confirms the crucial role cAMP-CRP plays in host survival and how this role is multi-faceted.

Much like these other host-bacterial relationships, *V. fischeri* requires certain colonization factors for host-cell attachment and successful initiation of colonization (126). Altura *et al.* provided evidence that during the early stages of *V. fischeri* colonization of *E. scolopes*, both attachment of *V. fischeri* to host cilia and aggregation between *V. fischeri* cells, which is mediated by exopolysaccharide production, were necessary for initiation (5). Yip *et al.* also showed the importance of *V. fischeri* cells aggregating with one another during initiation and successful colonization and identified a *syp* gene cluster in *V. fischeri* that is believed to be involved in biofilm formation and exopolysaccharide production (169, 170). Symbiotic colonization of *syp* mutants resulted in over a 1000-fold less CFU/squid than wild type colonized squid (169). In addition, a two-component regulator, RscS has a predicted role in regulating this *syp* gene cluster and a *rscS* mutant also has severe colonization defects (157, 169).

Such low colonization is comparable to the levels I saw with the Δ*cyaA* and Δ*crp* mutants (Fig 2.1), and I speculate that CRP-mediated regulation of *syp* and/or *rscS* may in part underlie the symbiotic phenotype of these mutants. Interestingly, a virtual footprint analysis (108) has

revealed a CRP site in the intergenic region between *rscS* and the divergent *glpR* although this site is closer to the *glpR* promoter, which is a likely target for cAMP-CRP, given its role in activating glycerol catabolism. In addition, putative CRP sites were also found in the *syp* gene cluster upstream of *sypG* and also *sypA*, the first gene in the operon. This 18-gene operon contains annotated glycosyltransferases and genes involved in polysaccharide metabolism. At this time, however, the reason for the severe attenuation of colonization by the Δ*cyaA* and Δ*crp* mutants is not clear. As noted in Chapter 2, the growth attenuation of these mutants in culture does not seem as severe as their attenuation in the squid. This hints at cAMP-CRP having a role in regulating an unknown colonization factor in addition to activating catabolic operons. Further work needs to be done to determine the CRP regulon in *V. fischeri*, and to determine if important colonization factors, such as the *syp* genes, are controlled by cAMP-CRP.

I also believe the relationship between cAMP-CRP and NAG catabolism should be explored further, because NAG may be a potential carbon source for *V. fischeri* in the squid. At present, the relationship between CRP and NAG is somewhat puzzling. In *V. cholerae*, the $EIIA^{Glc}$ component of the glucose PTS can transfer the phosphoryl group the NAG-specific EIIB domain and them to the incoming NAG (11, 66). Therefore, as with glucose, one would predict less activation of adenylate cyclase and lower cAMP production in the presence of NAG. Similarly, in *V. cholerae* both glucose and NAG can cause suppression of chitin degradation and the colonization of chitin surfaces, presumably through the reduction of cAMP-CRP levels (11). Consistent with this idea, *crp* and *cyaA* mutants of *V. cholerae* could not colonize chitin surfaces well. Likewise, we have evidence in *V. fischeri* that $EIIA^{Glc}$ may play role in the NAG PTS. Given the similarities between NAG and glucose, it is puzzling that our cAMP-CRP-dependent reporter showed elevated activity in the presence of NAG when glucose had the opposite effect.

On the other hand, from what I have shown in Chapter 3, the cAMP-CRP activity in *V. fischeri* is independent of cAMP levels, so NAG and glucose could have the same effect on cAMP while exerting different effects on cAMP-CRP activity.

In another connection between NAG and CRP, a virtual footprint analysis revealed a putative CRP binding site in the intergenic region of the divergent *nagE-nagAC* operon in *V. fischeri*. In *V. cholerae*, cAMP-CRP has different roles at this operon in which it (i) helps stabilize the repressor loop of NagC to repress this operon and (ii) activates transcription of these genes in the absence of NagC (56). Even if this is also true in *V. fischeri*, it remains difficult to interpret the role of cAMP-CRP in NAG catabolism.

Another important unresolved question is whether NAG is even physiologically relevant in the symbiosis. An important source of NAG is chitin, which is abundant in the marine environment, and is even found in *E. scolopes.* This squid produces its own chitin, which is present in the tissue lining the light-organ ducts (95). Once *V. fischeri* arrives at the pore to the light organ, it migrates through the duct down into the crypts. The migration towards the crypts is thought to be directed by a chemotactic response towards chitin oligosaccharides (95). *V. fischeri* had been shown to have a strong chemotactic response to NAG (33); however, it has been proposed that chitobiose, a disaccharide of glucosamine, is more physiologically relevant (95). Mandel *et al.* showed data supporting a model wherein *V. fischeri* degrades host-associated chitin, releasing chitobiose, and thus creating a gradient that *V. fischeri* will follow into the light organ (95). This model is further supported by the observed phenotype of a *cheA* mutant, which is chemotaxis-deficient, being defective in entering the light organ pores (95).

In Chapter 3, I further characterized the effect that carbon source has on the cAMP levels of the cell and on cAMP-CRP activity. What I found suggested that unlike the prevailing model

in *E. coli*, the cAMP levels in *V. fischeri* do not dictate cAMP-CRP activity at least under the conditions tested here. It should be noted that much of the previous work in *E. coli* should be cautiously interpreted, because some studies have correlated the native β-galactosidase activity of *E. coli* with cAMP levels, inferring that higher β-galactosidase activity equals higher cAMP-CRP activity and therefore higher cAMP levels (47, 61).

I sought to investigate this relationship between glucose and cAMP-CRP activity in *V. fischeri* by directly measuring cAMP levels and using an artificial cAMP-CRP-dependent reporter that is theoretically void of other intrinsic regulatory elements affecting reporter activity. Given that glucose still lowered CRP activity both in the absence of cAMP in the Δ*cyaA crp2* mutant and with removal of native transcriptional control, I hypothesize that protein acetylation is regulating CRP activity in the presence of glucose. As a good start to testing this hypothesis, proteomic analysis of *V. fischeri*'s CRP from cells grown on glycerol or glucose would be useful to determine if there are protein modifications and if these modifications are acetylated lysines. There is an established connection between CRP and acetylation, in that it has been shown in *E. coli* that CRP regulates proteins involved in acetylation.

In *E. coli*, CRP activates transcription of the acetyltransferase YfiQ (or PatZ), a homolog of *Salmonella*'s Pat acetyltransferase (25). In both *Salmonella* and *E. coli*, these homologs are responsible for inhibiting Acs activity by acetylation (25). In *E. coli*, a *patZ* mutant only had a slight growth defect on glucose (25), whereas in *Salmonella*, a *pat* mutant grew slower on glucose due to decreased acetylation (160). For *V. fischeri*, preliminary work with a *yfiQ* mutant did not reveal any growth defects in glucose, nor did I see increased cAMP-CRP-dependent reporter activity in the presence of glucose, indicating that *V. fischeri*'s YfiQ is an unlikely candidate for acetylating CRP. In addition to regulating PatZ, cAMP-CRP can also activate

transcription of *acs* (78), suggesting that both the acetyltransferase PatZ and Acs are expressed under the same conditions (25). This supports the notion that multiple regulatory mechanisms are in place to fine tune Acs activity by acetylation under conditions where cAMP-CRP would also activate transcription of *acs* (25). It is likely *V. fischeri* could have similar mechanisms in place to fine tune the enzymes in central metabolism both at the protein level and at the transcriptional level by cAMP-CRP.

A proteomic analysis of the CRP protein would help identify whether or not CRP is being acetylated, and this would be a step toward determining the physiological significance of CRP acetylation in *V. fischeri*. Not only could such post-translational modification of CRP affect the metabolic activity of the cell, but it could also affect pheromone-mediated signaling and most importantly, bioluminescence. Bioluminescence is lowered in the presence of glucose in *V. fischeri* and this could be accounted for by low cAMP-CRP activity (91). Taken together, metabolism and bioluminescence seem to be coordinated together by the effects on cAMP-CRP activity. If we can better determine how metabolism of certain carbon sources affect cAMP-CRP activity, then we can better understand how the environmental cues *V. fischeri* encounters during the symbiosis affects pheromone-mediated regulation.

REFERENCES

1. **Adin, D. M., K. L. Visick, and E. V. Stabb.** 2008. Identification of a cellobiose utilization gene cluster with cryptic beta-galactosidase activity in *Vibrio fischeri*. Appl. Environ. Microbiol. **74:**4059-4069.
2. **Aiba, H.** 1985. Transcription of the *Escherichia coli* adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. J. Biol. Chem. **260:**3063-3070.
3. **Albus, A. M., E. C. Pesci, L. J. Runyen-Janecky, S. E. West, and B. H. Iglewski.** 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3928-3935.
4. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
5. **Altura, M. A., E. A. Heath-Heckman, A. Gillette, N. Kremer, A. M. Krachler, C. Brennan, E. G. Ruby, K. Orth, and M. J. McFall-Ngai.** 2013. The first engagement of partners in the *Euprymna scolopes-Vibrio fischeri* symbiosis is a two-step process initiated by a few environmental symbiont cells. Environ. Microbiol.
6. **Amin, N., and A. Peterkofsky.** 1995. A dual mechanism for regulating cAMP levels in *Escherichia coli*. J. Biol. Chem. **270:**11803-11805.
7. **Antunes, L. C., A. L. Schaefer, R. B. Ferreira, N. Qin, A. M. Stevens, E. G. Ruby, and E. P. Greenberg.** 2007. Transcriptome analysis of the *Vibrio fischeri* LuxR-LuxI regulon. J. Bacteriol. **189:**8387-8391.

8. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi:* sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:**273-286.

9. **Bettenbrock, K., T. Sauter, K. Jahreis, A. Kremling, J. W. Lengeler, and E. D. Gilles.** 2007. Correlation between growth rates, EIIACrr phosphorylation, and intracellular cyclic AMP levels in *Escherichia coli* K-12. J. Bacteriol. **189:**6891-6900.

10. **Blattner, F. R., G. Plunkett, 3rd, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453-1462.

11. **Blokesch, M.** 2012. Chitin colonization, chitin degradation and chitin-induced natural competence of *Vibrio cholerae* are subject to catabolite repression. Environ. Microbiol. **14:**1898-1912.

12. **Boettcher, K. J., and E. G. Ruby.** 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J. Bacteriol. **172:**3701-3706.

13. **Boles, B. R., and A. R. Horswill.** 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. PLoS Path. **4:**e1000052.

14. **Bose, J. L., U. Kim, W. Bartkowski, R. P. Gunsalus, A. M. Overley, N. L. Lyell, K. L. Visick, and E. V. Stabb.** 2007. Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. Mol. Microbiol. **65:**538-553.

15. **Bose, J. L., C. S. Rosenberg, and E. V. Stabb.** 2008. Effects of *luxCDABEG* induction in *Vibrio fischeri:* enhancement of symbiotic colonization and conditional attenuation of growth in culture. Arch. Microbiol. **190:**169-183.

16. **Bose, J. L., M. S. Wollenberg, D. M. Colton, M. J. Mandel, A. N. Septer, A. K. Dunn, and E. V. Stabb.** 2011. Contribution of rapid evolution of the *luxR-luxI* intergenic region to the diverse bioluminescence outputs of *Vibrio fischeri* strains isolated from different environments. Appl. Environ. Microbiol. **77:**2445-2457.

17. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100-122.

18. **Boyer, M., and F. Wisniewski-Dye.** 2009. Cell-cell signalling in bacteria: not simply a matter of quorum. FEMS Microbiol. Ecol. **70:**1-19.

19. **Boylan, M., A. F. Graham, and E. A. Meighen.** 1985. Functional identification of the fatty acid reductase components encoded in the luminescence operon of *Vibrio fischeri*. J. Bacteriol. **163:**1186-1190.

20. **Busby, S., and R. H. Ebright.** 1999. Transcription activation by catabolite activator protein (CAP). J. Mol. Biol. **293:**199-213.

21. **Callahan, L. T., 3rd, and S. H. Richardson.** 1973. Biochemistry of *Vibrio cholerae* virulence. 3. Nutritional requirements for toxin production and the effects of pH on toxin elaboration in chemically defined media. Infect. Immun. **7:**567-572.

22. **Callahan, S. M., N. W. Cornell, and P. V. Dunlap.** 1995. Purification and properties of periplasmic 3':5'-cyclic nucleotide phosphodiesterase. A novel zinc-containing enzyme from the marine symbiotic bacterium *Vibrio fischeri*. J. Biol. Chem. **270:**17627-17632.

23. **Cao, X., S. V. Studer, K. Wassarman, Y. Zhang, E. G. Ruby, and T. Miyashiro.** 2012. The novel sigma factor-like regulator RpoQ controls luminescence, chitinase activity, and motility in *Vibrio fischeri*. mBio **3**.

24. **Castang, S., S. Reverchon, P. Gouet, and W. Nasser.** 2006. Direct evidence for the modulation of the activity of the *Erwinia chrysanthemi* quorum-sensing regulator ExpR by acylhomoserine lactone pheromone. J. Biol. Chem. **281:**29972-29987.

25. **Castano-Cerezo, S., V. Bernal, J. Blanco-Catala, J. L. Iborra, and M. Canovas.** 2011. cAMP-CRP co-ordinates the expression of the protein acetylation pathway with central metabolism in *Escherichia coli*. Mol. Microbiol. **82:**1110-1128.

26. **Chatterjee, J., C. M. Miyamoto, A. Zouzoulas, B. F. Lang, N. Skouris, and E. A. Meighen.** 2002. MetR and CRP bind to the *Vibrio harveyi lux* promoters and regulate luminescence. Mol. Microbiol. **46:**101-111.

27. **Chen, H. C., C. C. Chang, W. J. Mau, and L. S. Yen.** 2002. Evaluation of *N*-acetylchitooligosaccharides as the main carbon sources for the growth of intestinal bacteria. FEMS Microbiol. Lett. **209:**53-56.

28. **Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature **415:**545-549.

29. **Croda-Garcia, G., V. Grosso-Becerra, A. Gonzalez-Valdez, L. Servin-Gonzalez, and G. Soberon-Chavez.** 2011. Transcriptional regulation of *Pseudomonas aeruginosa rhlR*: role of the CRP orthologue Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhlR. Microbiology **157:**2545-2555.

30. **Cunningham, L., M. J. Gruer, and J. R. Guest.** 1997. Transcriptional regulation of the aconitase genes (*acnA* and *acnB*) of *Escherichia coli*. Microbiology **143 (Pt 12):**3795-3805.

31. **De Keersmaecker, S. C., K. Sonck, and J. Vanderleyden.** 2006. Let LuxS speak up in AI-2 signaling. Trends Microbiol. **14:**114-119.

32. **de Kievit, T. R., Y. Kakai, J. K. Register, E. C. Pesci, and B. H. Iglewski.** 2002. Role of the *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in *rhlI* regulation. FEMS Microbiol. Lett. **212:**101-106.

33. **DeLoney-Marino, C. R., A. J. Wolfe, and K. L. Visick.** 2003. Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and *N*-acetylneuraminic acid, a component of squid light-organ mucus. Appl. Environ. Microbiol. **69:**7527-7530.

34. **Deutscher, J., C. Francke, and P. W. Postma.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol. Rev. **70:**939-1031.

35. **Dunlap, P. V.** 1989. Regulation of luminescence by cyclic AMP in *cya*-like and *crp*-like mutants of *Vibrio fischeri*. J. Bacteriol. **171:**1199-1202.

36. **Dunlap, P. V., and S. M. Callahan.** 1993. Characterization of a periplasmic 3':5'-cyclic nucleotide phosphodiesterase gene, *cpdP*, from the marine symbiotic bacterium *Vibrio fischeri*. J. Bacteriol. **175:**4615-4624.

37. **Dunlap, P. V., and E. P. Greenberg.** 1985. Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic AMP and cyclic AMP receptor protein. J. Bacteriol. **164:**45-50.

38. **Dunlap, P. V., and E. P. Greenberg.** 1988. Control of *Vibrio fischeri lux* gene transcription by a cyclic AMP receptor protein-*luxR* protein regulatory circuit. J. Bacteriol. **170:**4040-4046.

39. **Dunn, A. K., M. O. Martin, and E. V. Stabb.** 2005. Characterization of pES213, a small mobilizable plasmid from *Vibrio fischeri*. Plasmid **54:**114-134.

40. **Dunn, A. K., D. S. Millikan, D. M. Adin, J. L. Bose, and E. V. Stabb.** 2006. New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression *in situ*. Appl. Environ. Microbiol. **72:**802-810.

41. **Dunn, A. K., and E. V. Stabb.** 2007. Beyond quorum sensing: the complexities of prokaryotic parliamentary procedures. Anal. Bioanal. Chem. **387:**391-398.

42. **Dunn, A. K., and E. V. Stabb.** 2008. Genetic analysis of trimethylamine *N*-oxide reductases in the light organ symbiont *Vibrio fischeri* ES114. J. Bacteriol. **190:**5814-5823.

43. **Eberhard, A.** 1972. Inhibition and activation of bacterial luciferase synthesis. J. Bacteriol. **109:**1101-1105.

44. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20:**2444-2449.

45. **Engebrecht, J., K. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773-781.

46. **Engebrecht, J., and M. Silverman.** 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. U. S. A. **81:**4154-4158.

47. **Epstein, W., L. B. Rothman-Denes, and J. Hesse.** 1975. Adenosine 3':5'-cyclic monophosphate as mediator of catabolite repression in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **72:**2300-2304.

48. **Feucht, B. U., and M. H. Saier, Jr.** 1980. Fine control of adenylate cyclase by the phosphoenolpyruvate:sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **141:**603-610.

49. **Fidopiastis, P. M., C. M. Miyamoto, M. G. Jobling, E. A. Meighen, and E. G. Ruby.** 2002. LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. Mol. Microbiol. **45:**131-143.

50. **Fong, J. C., and F. H. Yildiz.** 2008. Interplay between cyclic AMP-cyclic AMP receptor protein and cyclic di-GMP signaling in *Vibrio cholerae* biofilm formation. J. Bacteriol. **190:**6646-6659.

51. **Freeman, J. A., and B. L. Bassler.** 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. Mol. Microbiol. **31:**665-677.

52. **Freeman, J. A., and B. L. Bassler.** 1999. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. J. Bacteriol. **181:**899-906.

53. **Fuchs, E. L., E. D. Brutinel, A. K. Jones, N. B. Fulcher, M. L. Urbanowski, T. L. Yahr, and M. C. Wolfgang.** 2010. The *Pseudomonas aeruginosa* Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. J. Bacteriol. **192:**3553-3564.

54. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. **3:**685-695.

55. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269-275.

56. **Ghosh, S., K. H. Rao, M. Sengupta, S. K. Bhattacharya, and A. Datta.** 2011. Two gene clusters co-ordinate for a functional *N*-acetylglucosamine catabolic pathway in *Vibrio cholerae*. Mol. Microbiol. **80:**1549-1560.

57. **Gilson, L., A. Kuo, and P. V. Dunlap.** 1995. AinS and a new family of autoinducer synthesis proteins. J. Bacteriol. **177:**6946-6951.

58. **Gorke, B., and J. Stulke.** 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat. Rev. Microbiol. **6:**613-624.

59. **Graf, J., and E. G. Ruby.** 1998. Host-derived amino acids support the proliferation of symbiotic bacteria. Proc. Nat. Acad. Sci. U.S.A. **95:**1818-1822.

60. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

61. **Hantke, K., K. Winkler, and J. E. Schultz.** 2011. *Escherichia coli* exports cyclic AMP via TolC. J. Bacteriol. **193:**1086-1089.

62. **Hanzelka, B. L., and E. P. Greenberg.** 1996. Quorum sensing in *Vibrio fischeri*: evidence that *S*-adenosylmethionine is the amino acid substrate for autoinducer synthesis. J. Bacteriol. **178:**5291-5294.

63. **Hanzelka, B. L., A. M. Stevens, M. R. Parsek, T. J. Crone, and E. P. Greenberg.** 1997. Mutational analysis of the *Vibrio fischeri* LuxI polypeptide: critical regions of an autoinducer synthase. J. Bacteriol. **179:**4882-4887.

64. **Herrero, M., V. De Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. J. Bacteriol. **172:**6557-6567.

65. **Horton, R. M., Z. Cai, S. M. Ho, and L. R. Pease.** 2013. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **54:**129-133.

66. **Houot, L., S. Chang, C. Absalon, and P. I. Watnick.** 2010. *Vibrio cholerae* phosphoenolpyruvate phosphotransferase system control of carbohydrate transport, biofilm formation, and colonization of the germfree mouse intestine. Infect. Immun. **78:**1482-1494.

67. **Imamura, R., K. Yamanaka, T. Ogura, S. Hiraga, N. Fujita, A. Ishihama, and H. Niki.** 1996. Identification of the *cpdA* gene encoding cyclic 3',5'-adenosine monophosphate phosphodiesterase in *Escherichia coli*. J. Biol. Chem. **271:**25423-25429.

68. **Isaacs, H., Jr., D. Chao, C. Yanofsky, and M. H. Saier, Jr.** 1994. Mechanism of catabolite repression of tryptophanase synthesis in *Escherichia coli*. Microbiology **140 (Pt 8):**2125-2134.

69. **Ishizuka, H., A. Hanamura, T. Kunimura, and H. Aiba.** 1993. A lowered concentration of cAMP receptor protein caused by glucose is an important determinant for catabolite repression in *Escherichia coli*. Mol. Microbiol. **10:**341-350.

70. **Jones, B. W., and M. K. Nishiguchi.** 2004. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). Mar. Biol. **144:**1151-1155.

71. **Kanack, K. J., L. J. Runyen-Janecky, E. P. Ferrell, S. J. Suh, and S. E. West.** 2006. Characterization of DNA-binding specificity and analysis of binding sites of the

Pseudomonas aeruginosa global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein. Microbiology **152:**3485-3496.

72. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J. Bacteriol. **163:**1210-1214.

73. **Kim, H. S., S. M. Kim, H. J. Lee, S. J. Park, and K. H. Lee.** 2009. Expression of the *cpdA* gene, encoding a 3',5'-cyclic AMP (cAMP) phosphodiesterase, is positively regulated by the cAMP-cAMP receptor protein complex. J. Bacteriol. **191:**922-930.

74. **Kim, J., S. Adhya, and S. Garges.** 1992. Allosteric changes in the cAMP receptor protein of *Escherichia coli*: hinge reorientation. Proc. Nat. Acad. Sci. U.S.A. **89:**9700-9704.

75. **Kim, S. P., C. M. Kim, and S. H. Shin.** 2012. Cyclic AMP and cyclic AMP-receptor protein modulate the autoinducer-2-mediated quorum sensing system in *Vibrio vulnificus*. Curr. Microbiol. **65:**701-710.

76. **Kimbrough, J. H., and E. V. Stabb.** 2013. Substrate specificity and function of the pheromone receptor AinR in *Vibrio fischeri* ES114. J. Bacteriol. **195:**5223-5232.

77. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749-795.

78. **Kumari, S., C. M. Beatty, D. F. Browning, S. J. Busby, E. J. Simel, G. Hovel-Miner, and A. J. Wolfe.** 2000. Regulation of acetyl coenzyme A synthetase in *Escherichia coli*. J. Bacteriol. **182:**4173-4179.

79. **Kuo, A., N. V. Blough, and P. V. Dunlap.** 1994. Multiple *N*-acyl-L-homoserine lactone autoinducers of luminescence in the marine symbiotic bacterium *Vibrio fischeri*. J. Bacteriol. **176:**7558-7565.

80. **Kuo, A., S. M. Callahan, and P. V. Dunlap.** 1996. Modulation of luminescence operon expression by *N*-octanoyl-L-homoserine lactone in *ainS* mutants of *Vibrio fischeri*. J. Bacteriol. **178:**971-976.

81. **Lee, K. H., and E. G. Ruby.** 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. **60:**1565-1571.

82. **Lenz, D. H., K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen, and B. L. Bassler.** 2004. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. Cell **118:**69-82.

83. **Levy, S., G. Q. Zeng, and A. Danchin.** 1990. Cyclic AMP synthesis in *Escherichia coli* strains bearing known deletions in the *pts* phosphotransferase operon. Gene **86:**27-33.

84. **Liang, W., A. Pascual-Montano, A. J. Silva, and J. A. Benitez.** 2007. The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. Microbiology **153:**2964-2975.

85. **Liang, W., A. J. Silva, and J. A. Benitez.** 2007. The cyclic AMP receptor protein modulates colonial morphology in *Vibrio cholerae*. Appl. Environ. Microbiol. **73:**7482-7487.

86. **Liang, W., S. Z. Sultan, A. J. Silva, and J. A. Benitez.** 2008. Cyclic AMP post-transcriptionally regulates the biosynthesis of a major bacterial autoinducer to modulate the cell density required to activate quorum sensing. FEBS Lett. **582:**3744-3750.

87. **Lupp, C., and E. G. Ruby.** 2004. *Vibrio fischeri* LuxS and AinS: comparative study of two signal synthases. J. Bacteriol. **186:**3873-3881.

88. **Lupp, C., and E. G. Ruby.** 2005. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. J. Bacteriol. **187:**3620-3629.

89. **Lupp, C., M. Urbanowski, E. P. Greenberg, and E. G. Ruby.** 2003. The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol. Microbiol. **50:**319-331.

90. **Lupp, C., M. Urbanowski, E. P. Greenberg, and E. G. Ruby.** 2003. The Vibrio fischeri quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol. Microbiol. **50:**319-331.

91. **Lyell, N. L., D. M. Colton, J. L. Bose, M. P. Tumen-Velasquez, J. H. Kimbrough, and E. V. Stabb.** 2013. Cyclic AMP receptor protein regulates pheromone-mediated bioluminescence at multiple levels in *Vibrio fischeri* ES114. J. Bacteriol. **195:**5051-5063.

92. **Lyell, N. L., A. K. Dunn, J. L. Bose, and E. V. Stabb.** 2010. Bright mutants of *Vibrio fischeri* ES114 reveal conditions and regulators that control bioluminescence and expression of the *lux* operon. J. Bacteriol. **192:**5103-5114.

93. **Lyell, N. L., A. K. Dunn, J. L. Bose, S. L. Vescovi, and E. V. Stabb.** 2008. Effective mutagenesis of *Vibrio fischeri* by using hyperactive mini-Tn5 derivatives. Appl. Environ. Microbiol. **74:**7059-7063.

94. **Makman, R. S., and E. W. Sutherland.** 1965. Adenosine 3',5'-Phosphate in *Escherichia coli*. J. Biol. Chem. **240:**1309-1314.

95. **Mandel, M. J., A. L. Schaefer, C. A. Brennan, E. A. Heath-Heckman, C. R. Deloney-Marino, M. J. McFall-Ngai, and E. G. Ruby.** 2012. Squid-derived chitin

oligosaccharides are a chemotactic signal during colonization by *Vibrio fischeri*. Appl. Environ. Microbiol. **78:**4620-4626.

96. **McCann, J., E. V. Stabb, D. S. Millikan, and E. G. Ruby.** 2003. Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes*. Appl. Environ. Microbiol. **69:**5928-5934.

97. **McDougald, D., S. Srinivasan, S. A. Rice, and S. Kjelleberg.** 2003. Signal-mediated cross-talk regulates stress adaptation in *Vibrio* species. Microbiology **149:**1923-1933.

98. **Mcfall-Ngai, M., C. Brennan, V. Weis, and L. Lamarcq.,** (1998) Mannose adhesin-glycan interactions in the *Euprymna scolopes-Vibrio fischeri* symbiosis. In: New Developments in Marine Biotechnology. Y. Le Gal and H. O. Halvorson (eds). New York. Plenum Press, pp. 273-276

99. **McFall-Ngai, M. J., and E. G. Ruby.** 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science **254:**1491-1494.

100. **Meadow, N. D., D. K. Fox, and S. Roseman.** 1990. The bacterial phosphoenolpyruvate: glycose phosphotransferase system. Ann. Rev. of Biochem. **59:**497-542.

101. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, New York.

102. **Miyamoto, C. M., P. V. Dunlap, E. G. Ruby, and E. A. Meighen.** 2003. LuxO controls *luxR* expression in *Vibrio harveyi*: evidence for a common regulatory mechanism in *Vibrio*. Mol. Microbiol. **48:**537-548.

103. **Miyashiro, T., W. Klein, D. Oehlert, X. Cao, J. Schwartzman, and E. G. Ruby.** 2011. The *N*-acetyl-D-glucosamine repressor NagC of *Vibrio fischeri* facilitates colonization of *Euprymna scolopes*. Mol. Microbiol. **82:**894-903.

104. **Miyashiro, T., M. S. Wollenberg, X. Cao, D. Oehlert, and E. G. Ruby.** 2010. A single *qrr* gene is necessary and sufficient for LuxO-mediated regulation in *Vibrio fischeri*. Mol. Microbiol. **77:**1556-1567.

105. **More, M. I., L. D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard, and S. C. Winans.** 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. Science **272:**1655-1658.

106. **Mori, K., and H. Aiba.** 1985. Evidence for negative control of *cya* transcription by cAMP and cAMP receptor protein in intact *Escherichia coli* cells. J. Biol. Chem. **260:**14838-14843.

107. **Muller, C. M., A. Aberg, J. Straseviciene, L. Emody, B. E. Uhlin, and C. Balsalobre.** 2009. Type 1 fimbriae, a colonization factor of uropathogenic *Escherichia coli*, are controlled by the metabolic sensor CRP-cAMP. PLoS Path. **5:**e1000303.

108. **Munch, R., K. Hiller, A. Grote, M. Scheer, J. Klein, M. Schobert, and D. Jahn.** 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. Bioinformatics **21:**4187-4189.

109. **Nasser, W., M. L. Bouillant, G. Salmond, and S. Reverchon.** 1998. Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. Mol. Microbiol. **29:**1391-1405.

110. **Nealson, K. H.** 1977. Autoinduction of bacterial luciferase. Occurrence, mechanism and significance. Arch. Microbiol. **112:**73-79.

111. **Nielsen, L. D., D. Monard, and H. V. Rickenberg.** 1973. Cyclic 3',5'-adenosine monophosphate phosphodiesterase of *Escherichia coli*. J. Bacteriol. **116:**857-866.

112. **Ortori, C. A., S. Atkinson, S. R. Chhabra, M. Camara, P. Williams, and D. A. Barrett.** 2007. Comprehensive profiling of *N*-acylhomoserine lactones produced by *Yersinia pseudotuberculosis* using liquid chromatography coupled to hybrid quadrupole-linear ion trap mass spectrometry. Anal. Bioanal. Chem. **387:**497-511.

113. **Park, Y. H., B. R. Lee, Y. J. Seok, and A. Peterkofsky.** 2006. *In vitro* reconstitution of catabolite repression in *Escherichia coli*. J. Biol. Chem. **281:**6448-6454.

114. **Parsek, M. R., and E. P. Greenberg.** 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol. **13:**27-33.

115. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5'-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40:**527-551.

116. **Pastan, I., and R. Perlman.** 1970. Cyclic adenosine monophosphate in bacteria. Science **169:**339-344.

117. **Perlman, R. L., B. De Crombrugghe, and I. Pastan.** 1969. Cyclic AMP regulates catabolite and transient repression in *E. coli*. Nature **223:**810-812.

118. **Peterkofsky, A., and C. Gazdar.** 1974. Glucose inhibition of adenylate cyclase in intact cells of *Escherichia coli* B. Proc. Nat. Acad. Sci. U.S.A. **71:**2324-2328.

119. **Peterkofsky, A., Y. J. Seok, N. Amin, R. Thapar, S. Y. Lee, R. E. Klevit, E. B. Waygood, J. W. Anderson, J. Gruschus, H. Huq, and et al.** 1995. The *Escherichia coli* adenylyl cyclase complex: requirement of PTS proteins for stimulation by nucleotides. Biochemistry **34:**8950-8959.

120. **Plumbridge, J., and A. Kolb.** 1998. DNA bending and expression of the divergent *nagE-B* operons. Nucleic Acids Res. **26:**1254-1260.

121. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543-594.

122. **Qin, N., S. M. Callahan, P. V. Dunlap, and A. M. Stevens.** 2007. Analysis of LuxR regulon gene expression during quorum sensing in *Vibrio fischeri*. J. Bacteriol. **189:**4127-4134.

123. **Reddy, P., A. Peterkofsky, and K. McKenney.** 1985. Translational efficiency of the *Escherichia coli* adenylate cyclase gene: mutating the UUG initiation codon to GUG or AUG results in increased gene expression. Proc. Nat. Acad. Sci. U.S.A. **82:**5656-5660.

124. **Reverchon, S., M. L. Bouillant, G. Salmond, and W. Nasser.** 1998. Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. Mol. Microbiol. **29:**1407-1418.

125. **Reverchon, S., D. Expert, J. Robert-Baudouy, and W. Nasser.** 1997. The cyclic AMP receptor protein is the main activator of pectinolysis genes in *Erwinia chrysanthemi*. J. Bacteriol. **179:**3500-3508.

126. **Ruby, E. G.** 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu. Rev. Microbiol. **50:**591-624.

127. **Ruby, E. G., and K. H. Nealson.** 1977. Pyruvate production and excretion by the luminous marine bacteria. Appl. Environ. Microbiol. **34:**164-169.

128. **Ruby, E. G., and K. H. Nealson.** 1976. Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*; a model of symbiosis based on bacterial studies. Biol. Bull. **151:**574-586.

129. **Saier, M. H., Jr.** 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. Microbiol. Rev. **53:**109-120.

130. **Saier, M. H., Jr., B. U. Feucht, and L. J. Hofstadter.** 1976. Regulation of carbohydrate uptake and adenylate cyclase activity mediated by the enzymes II of the phosphoenolpyruvate: sugar phosphotransferase system in *Escherichia coli*. J. Biol. Chem. **251:**883-892.

131. **Saier, M. H., Jr., B. U. Feucht, and M. T. McCaman.** 1975. Regulation of intracellular adenosine cyclic 3':5'-monophosphate levels in *Escherichia coli* and *Salmonella typhimurium*. Evidence for energy-dependent excretion of the cyclic nucleotide. J. Biol. Chem. **250:**7593-7601.

132. **Saier, M. H., Jr., and S. Roseman.** 1976. Sugar transport. The *crr* mutation: its effect on repression of enzyme synthesis. J. Biol. Chem. **251:**6598-6605.

133. **Schaffer, A. A., L. Aravind, T. L. Madden, S. Shavirin, J. L. Spouge, Y. I. Wolf, E. V. Koonin, and S. F. Altschul.** 2001. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. **29:**2994-3005.

134. **Septer, A. N., Y. Wang, E. G. Ruby, E. V. Stabb, and A. K. Dunn.** 2011. The haem-uptake gene cluster in *Vibrio fischeri* is regulated by Fur and contributes to symbiotic colonization. Environ. Microbiol. **13:**2855-2864.

135. **Serate, J., G. P. Roberts, O. Berg, and H. Youn.** 2011. Ligand responses of Vfr, the virulence factor regulator from *Pseudomonas aeruginosa*. J. Bacteriol. **193:**4859-4868.

136. **Shadel, G. S., and T. O. Baldwin.** 1992. Positive autoregulation of the *Vibrio fischeri* *luxR* gene. LuxR and autoinducer activate cAMP-catabolite gene activator protein complex-independent and -dependent *luxR* transcription. J. Biol. Chem. **267:**7696-7702.

137. **Shadel, G. S., and T. O. Baldwin.** 1991. The *Vibrio fischeri* LuxR protein is capable of bidirectional stimulation of transcription and both positive and negative regulation of the *luxR* gene. J. Bacteriol. **173:**568-574.

138. **Shadel, G. S., R. Young, and T. O. Baldwin.** 1990. Use of regulated cell lysis in a lethal genetic selection in *Escherichia coli*: identification of the autoinducer-binding region of the LuxR protein from *Vibrio fischeri* ATCC 7744. J. Bacteriol. **172:**3980-3987.

139. **Singh, P., S. Ghosh, and A. Datta.** 2001. Attenuation of virulence and changes in morphology in *Candida albicans* by disruption of the *N*-acetylglucosamine catabolic pathway. Infect. Immun. **69:**7898-7903.

140. **Skorupski, K., and R. K. Taylor.** 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. Proc. Nat. Acad. Sci. U.S.A. **94:**265-270.

141. **Stabb, E. V.,** (2006) The *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. In: The Biology of Vibrios. F.L. Thompson, B. Austin and J. Swings (eds). Washington, D.C.: ASM Press, pp. 204-218

142. **Stabb, E. V., A. Schaefer, J. Bose, and E. G. Ruby.** (2008) Quorum signaling and symbiosis in the marine luminous bacterium *Vibrio fischeri*. In: Chemical Communication among Bacteria. Stephen C. Winans and Bonnie L. Bassler (eds). Washington, DC. ASM Press, pp. 233-250

143. **Stabb, E. V., and D.S. Millikan.** (2009) Is the *Vibrio fischeri-Euprymna scolopes* Symbiosis a Defense Mutualism? In: Defensive Mutualism in Microbial Symbiosis. James F. White Jr. and Monica S. Torres (eds). Boca Raton, FL. CRC Press, pp. 85-96

144. **Stabb, E. V., K. A. Reich, and E. G. Ruby.** 2001. *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD^+-glycohydrolases. J. Bacteriol. **183:**309-317.

145. **Stabb, E. V., and E. G. Ruby.** 2002. RP4-based plasmids for conjugation between *Escherichia coli* and members of the *Vibrionaceae*. Methods Enzymol. **358:**413-426.

146. **Stevens, A. M., and E. P. Greenberg.** 1997. Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. J. Bacteriol. **179:**557-562.

147. **Studer, S. V., M. J. Mandel, and E. G. Ruby.** 2008. AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. J. Bacteriol. **190:**5915-5923.

148. **Suh, S. J., L. J. Runyen-Janecky, T. C. Maleniak, P. Hager, C. H. MacGregor, N. A. Zielinski-Mozny, P. V. Phibbs, Jr., and S. E. West.** 2002. Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. Microbiology **148:**1561-1569.

149. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli, Salmonella typhimurium,* and *Vibrio harveyi:* a new family of genes responsible for autoinducer production. Proc. Nat. Acad. Sci. U.S.A. **96:**1639-1644.

150. **Takahashi, H., T. Inada, P. Postma, and H. Aiba.** 1998. CRP down-regulates adenylate cyclase activity by reducing the level of phosphorylated IIA(Glc), the glucose-specific phosphotransferase protein, in *Escherichia coli*. Mol. Gen. Genet. **259:**317-326.

151. **Timmen, M., B. L. Bassler, and K. Jung.** 2006. AI-1 influences the kinase activity but not the phosphatase activity of LuxN of *Vibrio harveyi*. J. Biol. Chem. **281:**24398-24404.

152. **Vendeville, A., K. Winzer, K. Heurlier, C. M. Tang, and K. R. Hardie.** 2005. Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nature Rev. Microbiol. **3:**383-396.

153. **Visick, K. L., J. Foster, J. Doino, M. McFall-Ngai, and E. G. Ruby.** 2000. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J. Bacteriol. **182:**4578-4586.

154. **Visick, K. L., and M. J. McFall-Ngai.** 2000. An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. J. Bacteriol. **182:**1779-1787.

155. **Visick, K. L., T. M. O'Shea, A. H. Klein, K. Geszvain, and A. J. Wolfe.** 2007. The sugar phosphotransferase system of *Vibrio fischeri* inhibits both motility and bioluminescence. J. Bacteriol. **189:**2571-2574.

156. **Visick, K. L., and E. G. Ruby.** 2006. *Vibrio fischeri* and its host: it takes two to tango. Curr. Opin. Microbiol. **9:**632-638.

157. **Visick, K. L., and L. M. Skoufos.** 2001. Two-component sensor required for normal symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. J. Bacteriol. **183:**835-842.

158. **Vogel, R. F., K. D. Entian, and D. Mecke.** 1987. Cloning and sequence of the *mdh* structural gene of *Escherichia coli* coding for malate dehydrogenase. Arch. Microbiol. **149:**36-42.

159. **Wang, L., Y. Hashimoto, C. Y. Tsao, J. J. Valdes, and W. E. Bentley.** 2005. Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in *Escherichia coli*. J. Bacteriol. **187:**2066-2076.

160. **Wang, Q., Y. Zhang, C. Yang, H. Xiong, Y. Lin, J. Yao, H. Li, L. Xie, W. Zhao, Y. Yao, Z. B. Ning, R. Zeng, Y. Xiong, K. L. Guan, S. Zhao, and G. P. Zhao.** 2010. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science **327:**1004-1007.

161. **West, S. E., A. K. Sample, and L. J. Runyen-Janecky.** 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J. Bacteriol. **176:**7532-7542.

162. **Whitehead, N. A., A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. **25:**365-404.

163. **Whiteley, M., and E. P. Greenberg.** 2001. Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. J. Bacteriol. **183:**5529-5534.

164. **Wier, A. M., S. V. Nyholm, M. J. Mandel, R. P. Massengo-Tiasse, A. L. Schaefer, I. Koroleva, S. Splinter-Bondurant, B. Brown, L. Manzella, E. Snir, H. Almabrazi, T. E. Scheetz, F. Bonaldo Mde, T. L. Casavant, M. B. Soares, J. E. Cronan, J. L. Reed, E. G. Ruby, and M. J. McFall-Ngai.** 2010. Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. Proc. Nat. Acad. Sci. U.S.A. **107:**2259-2264.

165. **Wilde, R. J., and J. R. Guest.** 1986. Transcript analysis of the citrate synthase and succinate dehydrogenase genes of *Escherichia coli* K12. J. Gen. Microbiol. **132:**3239-3251.

166. **Xavier, K. B., and B. L. Bassler.** 2003. LuxS quorum sensing: more than just a numbers game. Curr. Opin. Microbiol. **6:**191-197.

167. **Xavier, K. B., and B. L. Bassler.** 2005. Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. J. Bacteriol. **187:**238-248.

168. **Yang, J. K., and W. Epstein.** 1983. Purification and characterization of adenylate cyclase from *Escherichia coli* K12. J. Biol. Chem. **258:**3750-3758.

169. **Yip, E. S., K. Geszvain, C. R. DeLoney-Marino, and K. L. Visick.** 2006. The symbiosis regulator RscS controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. Mol. Microbiol. **62:**1586-1600.

170. **Yip, E. S., B. T. Grublesky, E. A. Hussa, and K. L. Visick.** 2005. A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. Mol. Microbiol. **57:**1485-1498.

171. **You, C., H. Okano, S. Hui, Z. Zhang, M. Kim, C. W. Gunderson, Y. P. Wang, P. Lenz, D. Yan, and T. Hwa.** 2013. Coordination of bacterial proteome with metabolism by cyclic AMP signalling. Nature **500:**301-306.

172. **Youn, H., R. L. Kerby, M. Conrad, and G. P. Roberts.** 2006. Study of highly constitutively active mutants suggests how cAMP activates cAMP receptor protein. J. Biol. Chem. **281:**1119-1127.

173. **Zhu, J., and S. C. Winans.** 1999. Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters *in vitro* and decreases TraR turnover rates in whole cells. Proc. Nat. Acad. Sci. U.S.A. **96:**4832-4837.