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REVIEW

Rethinking the roles of CRP, cAMP, and sugar-mediated global regulation in the *Vibrionaceae*

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Abstract Many proteobacteria modulate a suite of catabolic genes using the second messenger cyclic 3′, 5′-AMP (cAMP) and the cAMP receptor protein (CRP). Together, the cAMP-CRP complex regulates target promoters, usually by activating transcription. In the canonical model, the phosphotransferase system (PTS), and in particular the EIIA^{Glc} component for glucose uptake, provides a mechanistic link that modulates cAMP levels depending on glucose availability, resulting in more cAMP and activation of alternative catabolic pathways when glucose is unavailable. Within the *Vibrionaceae*, cAMP-CRP appears to play the classical role in modulating metabolic pathways; however, it also controls functions involved in natural competence, bioluminescence, pheromone signaling, and colonization of animal hosts. For this group of marine bacteria, chitin is an ecologically relevant resource, and chitin's monomeric sugar *N*-acetylglucosamine (NAG) supports robust growth while also triggering regulatory responses. Recent studies with *Vibrio fischeri* indicate that NAG and glucose uptake share EIIA^{Glc}, yet the responses of cAMP-CRP to these two carbon sources are starkly different. Moreover, control of cAMP levels appears to be more dominantly controlled by export and degradation. Perhaps more surprisingly, although CRP may require cAMP, its activity can be controlled in response to glucose by a mechanism independent of cAMP levels. Future studies in this area promise to shed new light on the role of cAMP and CRP.

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Introduction

The second messenger cyclic 3′, 5′-AMP (cAMP) together with the cAMP receptor protein (CRP) constitutes an archetype bacterial global regulator that contributes to the phenomenon of catabolite repression, wherein the lack of a preferred carbon source (e.g., glucose) induces catabolic pathways for growth on alternative substrates. Some of these catabolic pathways require cAMP-CRP for transcriptional activation (Gorke and Stulke 2008; Meadow et al. 1990; Postma et al. 1993; Botsford and Harman 1992). In the absence of glucose, cAMP levels are high and bind to CRP to alter its conformation, and together the cAMP-CRP duo binds DNA at specific palindromic sequences in target promoters to affect transcription of catabolic genes (Kolb et al. 1993; Busby and Ebright 1999).

E. coli as a model of CRP- and cAMP-mediated regulation

The control of cAMP-CRP and its role in catabolite repression have been studied most thoroughly in *Escherichia coli,* where the paradigm model is that cAMP-CRP activity is controlled largely by cAMP levels. In *E. coli*, adenylate cyclase (CyaA) generates cAMP (Yang and Epstein 1983), and CyaA activity is connected to glucose by this sugar's phosphotransferase system (PTS) EIIA^{Glc} component, which when phosphorylated stimulates CyaA to synthesize cAMP (Levy et al. 1990). When glucose is available, it is transported into the cell by the PTS EIIC domain and phosphorylated by the EIIB domain, which receives the phosphate from EIIA^{Glc}, thus leaving less phosphorylated

EIIA^{Glc} to stimulate cAMP production by CyaA (Amin and Peterkofsky 1995; Bettenbrock et al. 2007; Feucht and Saier 1980; Peterkofsky et al. 1995; Takahashi et al. 1998; Levy et al. 1990; Saier et al. 1976). Homeostasis of cAMP is also maintained by counterbalancing CyaA activity with cAMP turnover via the cAMP phosphodiesterase CpdA (Imamura et al. 1996) and/or with cAMP export (Saier et al. 1975). Recent studies, including one combining global "omics" measurements with mathematical modeling, suggest that cAMP-CRP activity is tied to availability of metabolic precursors and "metabolic need" (You et al. 2013), although the mechanisms involved remain elusive. As discussed below, our studies with *Vibrio fischeri* have in many ways paralleled the model derived from *E. coli*; however, neither "metabolic need" nor EIIA^{Glc}'s role in cAMP synthesis fully explain our observations.

Roles of CRP within the *Vibrionaceae*

Within the *Vibrionaceae*, which like *E. coli* are gammaproteobacteria, the CRP protein is ~95 % identical to CRP in *E. coli*, and *crp* can functionally complement between these genera (Lyell et al. 2013). There is ample evidence that CRP plays a classical role in regulating catabolic genes in the *Vibrionaceae*. For example, bioinformatic predictions of CRP-binding sites indicate they are often located upstream of catabolic operons, and experimental data have suggested or validated the role of cAMP-CRP in some of these catabolic pathways (Adin et al. 2008; Kim et al. 2011). Recently, phenotype microarray screening validated the role of *crp* in the utilization of several carbon sources by *Vibrio cholerae* (Chen et al. 2013). Furthermore, the observation that *crp* mutants grow relatively well on glucose but poorly if at all on other carbon sources is consistent with a role of cAMP-CRP in directing catabolism of non-glucose carbon sources (Colton et al. 2015; Lyell et al. 2013).

As is the case in other bacteria, cAMP-CRP can also direct regulatory changes associated with virulence both in *V. cholerae* [for a review, see (McDonough and Rodriguez 2012)] and *Vibrio vulnificus* (Kim et al. 2013). In addition to a long-established role in regulating virulence factors (Skorupski and Taylor 1997), cAMP-CRP has more recently been implicated in these *Vibrio* pathogens as regulating such varied activities as motility (Liang et al. 2007), biofilm formation (Fong and Yildiz 2008), pheromone signaling (Kim et al. 2012; Liang et al. 2007, 2008), natural competence (Antonova et al. 2012; Blokesch 2012), osmotolerance (Lee and Choi 2006), resistance to bacteriophages (Zahid et al. 2010), and integrase activity (Baharoglu et al. 2012). Although some of these behaviors, notably the uptake of foreign DNA, could be interpreted as nutritional responses, these observations illustrate the pervasive

rethinking of cAMP-CRP as a means to not only link nutritional status to nutritional responses but also to regulate appropriate behaviors based on the nutrient profile in a particular environment. This line of thinking reflects a larger trend wherein the regulatory responses to second messengers and phenomena like catabolite repression are being viewed more broadly, beyond the more narrowly defined behaviors initially described [e.g., (Bordeleau and Burrus 2015; Yang and Lan 2015)].

Sweetness and light: glucose, bioluminescence, and CRP in the symbiont *Vibrio fischeri*

We became interested in CRP in the bioluminescent light-organ symbiont *V. fischeri*, because of its reported role in regulating the *lux* genes underlying pheromone signaling and bioluminescence. The symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes,* has become a powerful model for studying persistent and mutualistic animal-bacteria associations (Stabb and Visick 2013; Stabb 2006), and bioluminescence is a colonization factor required for symbiotic persistence (Bose et al. 2008; Visick et al. 2000; Koch et al. 2014). How bioluminescence benefits the colonizing bacteria is unknown (Stabb 2005; Walker et al. 2006), but studies of its regulation in response to environmental cues could suggest conditions where it is advantageous (Bose et al. 2007; Lyell et al. 2010, 2013; Septer et al. 2010, 2013, 2014; Septer and Stabb 2012; Stabb and Flores-Cruz 2013; Cao et al. 2012; Whistler and Ruby 2003).

It is well established that bioluminescence is repressed by glucose (Friedrich and Greenberg 1983), and several studies suggested the involvement of cAMP and CRP (Dunlap 1989; Dunlap and Greenberg 1985, 1988; Shadel et al. 1990). We recently validated and extended these studies in *V. fischeri* ES114, showing that the two acyl-homoserine lactone pheromone-signaling systems that control bioluminescence are both activated by cAMP-CRP (Lyell et al. 2013). Bioluminescence is induced early in symbiotic colonization, and we recently showed that cAMP-CRP is active in this time period and also contributes to symbionts ability to colonize the host (Colton et al. 2015). We therefore became interested in validating a model of how cAMP and CRP functioned, based upon the *E. coli* paradigm described above.

Control of cAMP-CRP activity in *V. fischeri* is the same only different

Upon comparing cAMP-CRP activity and cAMP levels in *V. fischeri* mutants and wild-type under different growth conditions, a model (Fig. 1) emerged that is both similar and different from our original hypotheses (Colton
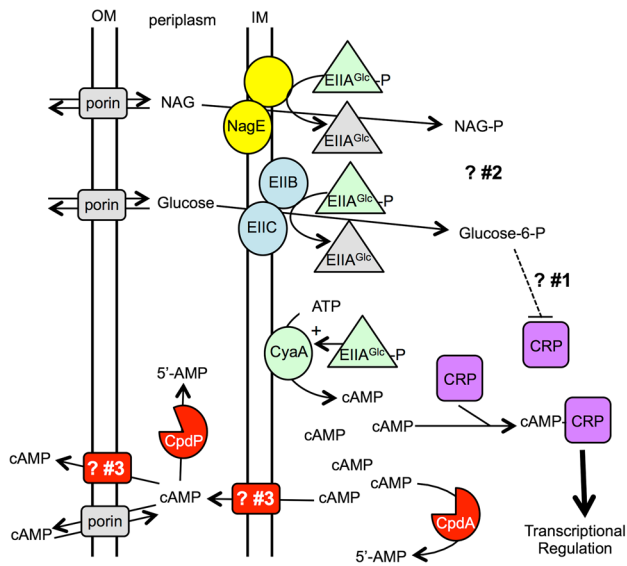
Fig. 1 Working model of cAMP-CRP control in *Vibrio fischeri*. CRP (*purple*) combines with cAMP and then binds DNA to regulate target promoters. Generation of cAMP (*green*) is accomplished by CyaA, which is stimulated by phosphorylated EIIA^{Glc}. Turnover of cAMP (*red*) involves intracellular degradation by CpdA, extracellular degradation by CpdP, and excretion by unknown transporters. Glucose and NAG are transported via dedicated PTS systems, EIIBC (PtsG) and NagE1, respectively, which apparently share substrate phosphorylation by EIIA^{Glc}. Three unresolved mysteries include: *First* (*? #1*), availability of glucose can modulate CRP activity independently of CyaA-determined cAMP levels, possibly by post-translational modification. *Second* (*? #2*), like glucose, NAG requires EIIA^{Glc} to be utilized, and NAG is presumably quickly processed to glucose-6-P, yet NAG and glucose have opposite effects on cAMP-CRP activity. *Third*, (*? #3*) cAMP is transported either into the periplasm or out of the cell, and the amount of extracellular cAMP seems to vary with growth substrate, but no mechanisms for transport or its control have been identified. Inner membrane (*IM*) and outer membrane (*OM*)

et al. 2015). It should be noted that as in all studies, our results are only valid under the specific set of conditions we tested. The relative importance of various mechanisms controlling cAMP-CRP could well be context dependent, and our interpretations cannot be extrapolated to all conditions. Similarly, it remains unclear the extent to which our data reflect differences between *E. coli* and *V. fischeri* as opposed to differences in the conditions of specific experiments. Studying cAMP-CRP also poses some experimental difficulties. For example, transcriptional reporters of cAMP-CRP-regulated genes are generally also controlled by other regulators, a complication that we avoided by generating a synthetic cAMP-CRP-dependent promoter (Colton et al. 2015). On the other hand, we found no workable solution for our observation that cAMP present in yeast extract interfered with attempts to measure *Vibrio*-derived extracellular cAMP when cells were grown in our complex and rich media amended with various carbon sources. Given the many ways cells can be grown and the

various measurements that can or cannot be made in vivo or in vitro, with their respective limitations interpreting, and reconciling the literature in this field can be challenging.

With those caveats in mind, in certain respects, our data were consistent with our understanding of cAMP-CRP's function. Most importantly, cAMP-CRP activity was relatively low in media supplemented with glucose. Our data were also consistent with a model whereby CyaA was responsible for cAMP generation, EIIA^{Glc} could enhance cAMP production, CpdA consumed intracellular cAMP, and some cAMP was exported from the cell. Each of these findings is consistent with the model described above derived from our understanding of *E. coli*.

We also knew that *V. fischeri* is endowed with a periplasmic cAMP phosphodiesterase, named CpdP (Dunlap and Callahan 1993; Callahan et al. 1995), and this effectively prevented the accumulation of extracellular cAMP (Colton et al. 2015). Although this role for CpdP was not unexpected, it does cast the function of CpdP in a new light. Initially discovered and characterized in the context of enabling growth on exogenous cAMP as a sole source of carbon and energy, Dunlap and Callahan (1993) also speculated that CpdP might separate cAMP production and turnover into different compartments. Our data indicate that CpdP does not replace the cytoplasmic turnover of cAMP by CpdA; however, it could provide a way to turn over and recycle endogenously produced cAMP that other bacteria would lose upon exporting it.

Despite similarities to the model based on *E. coli*, the relative contributions of the components shown in Fig. 1 were unexpected. In particular, the influence of EIIA^{Glc}, which in theory is the main connection between carbon source and cAMP-CRP activity, was subtle and only evident in *cpdA* mutants, which have elevated cAMP. Instead, it appears that intracellular levels of cAMP were determined more by its export and turnover, and that export of cAMP is somehow influenced by carbon source (Fig. 1). Our data indicated that while more cAMP was generated by cells growing on glycerol, more of it was exported too (Colton et al. 2015).

By far the most startling deviation from the paradigm model was our observation that intracellular cAMP levels were lower in cells grown on glycerol relative to cells grown with glucose, even though our cAMP-CRP-dependent reporter indicated higher activity under the same conditions (Colton et al. 2015). The latter observation is more consistent with all of our other data, which suggest a classical role of increased cAMP-CRP activity when glucose is unavailable. Moreover, using a CRP* variant, which does not require cAMP for activity, in a background lacking *cyaA* (and presumably cAMP), we still saw modulation of CRP* activity in response to glucose. Other experiments suggested this response is not due to transcriptional

regulation of *crp* or changes in CRP levels as described in *E. coli* (Ishizuka et al. 1993). As discussed below, we hypothesize that CRP might be post-translationally regulated (Fig. 1). Thus, taken together, our data suggest that cAMP levels in *V. fischeri* are modulated in ways that are both understood and not; however, it seems likely that cAMP-CRP activity can be altered in response to glucose via a cAMP-independent mechanism.

Other NAGging questions

Within the *Vibrionaceae*, which are predominately marine bacteria, the oligosaccharide chitin is an ecologically relevant resource, as is chitin's monomeric sugar *N*-acetylglucosamine (NAG). Chitin is a major structural component of many marine invertebrates, and the pathways for chitin and NAG utilization appear widespread and conserved within the *Vibrionaceae* (Hunt et al. 2008). In *V. fischeri*, it appears that chitin oligosaccharides and NAG play a role in nutrient provisioning from host to symbiont that is initially absent but ultimately develops and becomes part of the diurnal cycle of the adult host squid (Miyashiro et al. 2011; Schwartzman et al. 2015; Sun et al. 2015; Wier et al. 2010). Like glucose, NAG supports robust growth of many *Vibrio* species while also triggering regulatory responses. It is certainly true that NAG supports rapid growth of *V. fischeri*, and NAG resembles glucose in largely restoring growth to *crp* or *cya* mutants of *V. fischeri* (Colton et al. 2015; Lyell et al. 2013). As a carbon source, NAG seems no less "preferred" than glucose.

Pan et al. recently showed that the EIIA^{Glc} component of glucose transport is shared by, and required for, NAG import and utilization in *V. fischeri* (Pan et al. 2015), which is consistent with our own observations under different conditions. Moreover, EIIA^{Glc} and NAG appeared to function in the phenomenon of "inducer exclusion," an aspect of catabolite repression, whereby uptake of alternative carbon sources is attenuated by protein–protein interactions in the presence of a preferred carbon source. Yet despite the parallels between glucose and NAG, we found that the activity of cAMP-CRP was high in cells grown on NAG or glycerol, in contrast to low activity during growth on glucose (Colton et al. 2015) and (Fig. 2). Neither nutritional status nor the shared EIIA^{Glc} seems to account for the difference between NAG and glucose with respect to cAMP-CRP activity.

Perhaps, in certain members of the *Vibrionaceae*, glucose and NAG are both preferred carbon sources that share features such as the PTS component, EIIA^{Glc}, yet they represent different environments and niches and therefore elicit different regulatory responses. For example, NAG-specific regulation by transcriptional regulator NagC can mediate some differences in gene regulation between
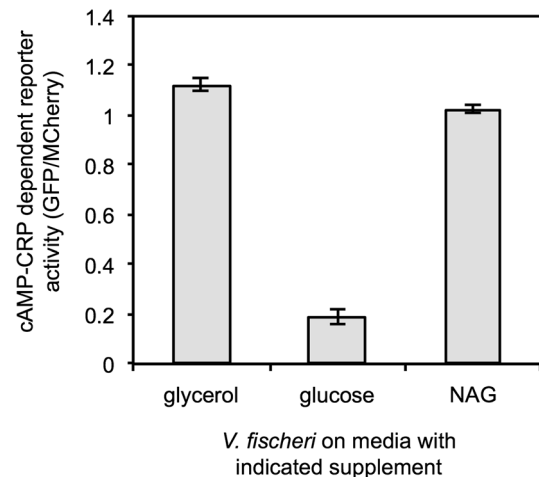

Fig. 2 cAMP-CRP activity in *V. fischeri* grown on media supplemented with glycerol, glucose, or NAG. Activity of the cAMP-CRP activated *gfp* transcriptional reporter on pDC85 (Colton et al. 2015) reported as green (GFP) fluorescence normalized against the red fluorescence of a constitutive mCherry on the same plasmid. The fluorescence ratios are averaged between three independent experiments for each condition, and bars indicate *standard error*. Cells were grown on plates of SWTO medium supplemented with glycerol, glucose, or NAG (Colton et al. 2015). The reporter activity in cells grown on glucose is significantly different from those grown on media supplemented with glycerol or NAG ($p < 0.01$). There is no significant difference in reporter activity in cells grown on media supplemented with NAG or glycerol ($p > 0.5$)

glucose- and NAG-grown cells (Ghosh et al. 2011). If the global effect of glucose was preserved in the cAMP-CRP response, it would make sense to evolve a system of cAMP-CRP control that de-emphasized the role of EIIA^{Glc}, so that the glucose response could remain distinct from NAG. How cAMP-CRP activity became connected to glucose but not NAG availability remains to be discovered.

Future directions

Chitin and NAG are important resources for *Vibrio* species, and additionally glucose is known to underpin many key regulatory responses in these bacteria. The recent studies discussed above and others suggest that much remains to be learned about cAMP and CRP in the *Vibrionaceae*. Although catabolite repression, the effects of cAMP-CRP, and responses to glucose are known to involve more than EIIA^{Glc} and cAMP levels (You et al. 2013; Ishizuka et al. 1993; Magasanik 1961), the recent results in *V. fischeri* are intriguing and suggest that this organism might be a good model for uncovering new connections between carbon source, cAMP, and CRP activity.

One priority for future experimentation should be to investigate the possible post-translational modification of CRP. As we discussed elsewhere (Colton et al. 2015),

a potential mechanism for such control would be lysine acetylation. In *E. coli*, CRP can be acetylated (Kuhn et al. 2014), and the acetylation levels of metabolic enzymes can depend on whether cells are growing on glucose or other carbon sources (Wang et al. 2010). If the analysis of the CRP protein harvested from cells grown on different carbon sources does show differences in mass, this would raise several questions, potentially including whether the decorations are added enzymatically or directly through acetyl-phosphate, whether they affect DNA binding or interactions with RNAP polymerase, and whether they act in a promoter-specific fashion.

Another unresolved question is how cAMP is exported. This phenomenon seems to be a common feature of bacteria that remains poorly understood. Although there is a report that the outer membrane channel protein TolC mediates cAMP export (Hantke et al. 2011), *tolC* did not influence extracellular cAMP in *V. fischeri*. Moreover, the results in *E. coli* seem open to other interpretations. We attempted without success to isolate mutants of *V. fischeri* that failed to export cAMP, but perhaps more sensitive or clever high-throughput genetic screens could be developed.

Given that NAG and glucose promote growth similarly and both require the EIIA^{Glc} PTS component, yet have radically different effects on cAMP-CRP activity, it should be interesting to investigate further the similarities and differences between utilization of these two sugars. Metabolic, transcriptomic, and proteomic profiles of wild-type and *crp* mutant cells grown on NAG versus glucose might provide valuable insight into both the different regulatory responses to these carbon sources and the mechanisms underpinning those responses. Such approaches could be particularly powerful if combined with follow-up experiments in select mutants, to test specific mechanistic hypotheses.

Investigations of regulatory responses to other carbon sources could also be useful. Notably, there are intriguing links to CRP activity and utilization of cellobiose, which is a dimer of beta-linked glucose units. *V. fischeri* can utilize cellobiose, there is a CRP-binding site upstream of the *cel* operon for cellobiose catabolism, and glucose leads to lowered expression from this promoter (Adin et al. 2008). The cellobiose PTS has its own EIIA component (CelA), the *cel* operon also encodes a glucokinase, and cellobiose utilization does not require EIIA^{Glc}, yet results suggest that the breakdown of cellobiose into glucose units leads to lowered expression from the *cel* promoter reminiscent of growth on glucose (Adin et al. 2008). Thus, cellobiose may represent a mechanism for affecting CRP activity with the carbon entering the cells through a different path than monomeric glucose.

Finally, the roles of EIIA^{Glc} seem to include both modulating cAMP levels and inducer exclusion, but the extent of its function remains to be explored. Notably, mutants disrupting the *crr* gene that encodes EIIA^{Glc} have

brighter bioluminescence than wild-type (Visick et al. 2007), and nothing in our understanding of cAMP-CRP, EIIA^{Glc}, or luminescence regulation seems to explain this phenomenon.

These studies and others in *V. fischeri* along with the ongoing investigations of CRP-mediated regulation and catabolite repression in other members of the *Vibrionaceae*, promise to expand our view of this global regulatory system.

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