Molecular Microbiology (2015) 97(6), 1114–1127
doi:10.1111/mmi.13087
First published online 17 July 2015

Growth on glucose decreases cAMP-CRP activity while paradoxically increasing intracellular cAMP in the light-organ symbiont *Vibrio fischeri*

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Summary

Proteobacteria often co-ordinate responses to carbon sources using CRP and the second messenger cyclic 3′, 5′-AMP (cAMP), which combine to control transcription of genes during growth on non-glucose substrates as part of the catabolite-repression response. Here we show that cAMP-CRP is active and important in *Vibrio fischeri* during colonization of its host squid *Euprymna scolopes*. Moreover, consistent with a classical role in catabolite repression, a cAMP-CRP-dependent reporter showed lower activity in cells grown in media amended with glucose rather than glycerol. Surprisingly though, intracellular cAMP levels were higher in glucose-grown cells. Mutant analyses were consistent with predictions that CyaA was responsible for cAMP generation, that the $EIIA^{Glc}$ component of glucose transport could enhance cAMP production and that the phophodiesterases CpdA and CpdP consumed intracellular and extracellular cAMP respectively. However, the observation of lower cAMP levels in glycerol-grown cells seemed best explained by changes in cAMP export, via an unknown mechanism. Our data also indicated that cAMP-CRP activity decreased during growth on glucose independently of *crp*'s native transcriptional regulation or cAMP levels. We speculate that some unknown mechanism, perhaps carbon-source-dependent post-translational modulation of CRP, may help control cAMP-CRP activity in *V. fischeri*.

Introduction

Vibrio fischeri is a bioluminescent bacterium that forms light-organ symbioses with marine animals, including the Hawaiian bobtail squid, *Euprymna scolopes* (Stabb and Visick, 2013), and this symbiosis is a powerful model for studying host-associated bacteria and their regulatory responses (Ruby, 1996; Stabb, 2006). Two areas of particular interest have been the growth substrates available to symbionts and the induction of bioluminescence, the latter drawing interest partly because it is governed by pheromone-mediated regulation.

The second messenger cyclic 3′, 5′-AMP (cAMP) together with the cAMP-receptor protein (CRP) were among the first discovered regulators of luminescence (Dunlap and Greenberg, 1985; 1988; Dunlap, 1989), and luminescence is repressed by glucose (Friedrich and Greenberg, 1983). cAMP-CRP is widespread among the Proteobacteria and is best known for activating expression of catabolic pathways in response to growth on non-preferred (e.g. non-glucose) substrates (Meadow *et al.*, 1990; Botsford and Harman, 1992; Postma *et al.*, 1993; Gorke and Stulke, 2008). cAMP binds to CRP and alters its conformation such that cAMP-CRP binds DNA and activates, or less frequently inhibits, transcription (Kolb *et al.*, 1993; Busby and Ebright, 1999).

The modulation of cAMP-CRP in response to growth substrate has been elucidated mainly in *Escherichia coli*, where the model has emerged that cAMP-CRP activity is controlled largely by cAMP levels. Adenylate cyclase (CyaA) generates cAMP (Yang and Epstein, 1983), and a connection to glucose availability is provided by the glucose phosphotransferase system $EIIA^{Glc}$ component, the phosphorylated form of which stimulates CyaA activity (Levy *et al.*, 1990). When glucose is available, it is phosphorylated upon transport into the cell, with the consequence of less phosphorylated $EIIA^{Glc}$ and therefore less CyaA activity and decreased cAMP synthesis (Saier *et al.*, 1976; Feucht and Saier, 1980; Levy *et al.*, 1990; Amin and Peterkofsky, 1995; Peterkofsky *et al.*, 1995; Takahashi *et al.*, 1998; Bettenbrock *et al.*, 2007).

Other controls over cAMP include cAMP export and cAMP turnover, the latter of which is accomplished by CpdA (Imamura *et al.*, 1996; Kim *et al.*, 2009). Interestingly, *V. fischeri* possesses both CpdA and a periplasmic cAMP phosphodiesterase CpdP, which is absent from *E. coli* (Dunlap and Callahan, 1993; Callahan *et al.*, 1995). Bacteria also release cAMP, which could be an important control over intracellular cAMP (Hantke *et al.*, 2011). Finally, cAMP-CRP activity may be modulated by other

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mechanisms, such as the control of CRP levels (Ishizuka *et al*., 1993), and it is worth noting that while changes in cAMP-CRP activity are often attributed to cAMP levels, frequently this model is assumed rather than measured.

Within the *Vibrionaceae*, CRP plays the traditional role in regulating catabolic genes, but it also controls other processes including motility, virulence and pheromone-mediated signaling (Skorupski and Taylor, 1997; Silva and Benitez, 2004; Liang *et al*., 2007; Fong and Yildiz, 2008; Kim *et al*., 2012; 2013). We recently found that in *V. fischeri*, CRP regulates both the AinS/AinR and LuxI/LuxR pheromone systems (Lyell *et al*., 2013), which in turn regulate colonization factors such as motility and luminescence (Engebrecht and Silverman, 1984; Graf *et al*., 1994; Visick *et al*., 2000; Lupp and Ruby, 2005; Bose *et al*., 2008). The activity of cAMP-CRP during host colonization is thought to be related to carbon source; however, the growth substrates accessed by symbiotic *V. fischeri* are not fully defined. Nealson and Ruby suggested that glucose metabolism might play a role for *V. fischeri* symbionts in fish light organs (Ruby and Nealson, 1977), although that host was not experimentally tractable. In the *E. scolopes* symbiosis, host epithelial cells slough into the light-organ crypts, providing complex growth substrates (Ruby, 1996), and *V. fischeri* symbionts can access amino acids, mostly in the form of peptides (Graf and Ruby, 1998). Other substrates that *V. fischeri* may use include mannose, chitin, *N*-acetylglucosamine (NAG), glycerol and glycerol-3-phosphate (McFall-Ngai *et al*., 1998; Wier *et al*., 2010). A *nagB* mutant impaired in NAG utilization showed no apparent symbiotic defect (Miyashiro *et al*., 2011), although a *ptsI* mutant affected in the utilization of several sugars, including glucose, was severely impaired in colonization (Adin *et al*., 2008). Despite what is known, the nutritional status of *V. fischeri* symbionts requires further investigation.

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

Results

cAMP-CRP is active and important during host colonization

To assess cAMP-CRP's role during the squid symbiosis, we assayed the ability of Δ*cyaA* and Δ*crp* mutants to colonize aposymbioitc squid. In our first experiment, at a comparable inoculum density of ~2000 CFU ml^{-1}, squid infection was undetectable with a Δ*crp* mutant, whereas wild-type ES114 infected and colonized all the inoculated animals (data not shown). When the Δ*crp* mutant inoculum was increased to ~4-fold more than that of wild type, the mutant colonized the squid but only achieved populations 200-fold less than that of wild type after 24 h, and over 1000-fold less than wild type at 48 h (Fig. 1A). We saw similar colonization defects with the Δ*cyaA* mutant. For example, Fig. 1B shows a typical experiment where at an inoculum density ~2000 CFU ml^{-1} only three out of the 11 animals were detectably colonized by the mutant, and even those animals harbored much lower numbers of *V. fischeri* than did hatchlings infected with ES114. Thus, both *cyaA* and *crp* are apparently required for robust colonization initiation and persistence.

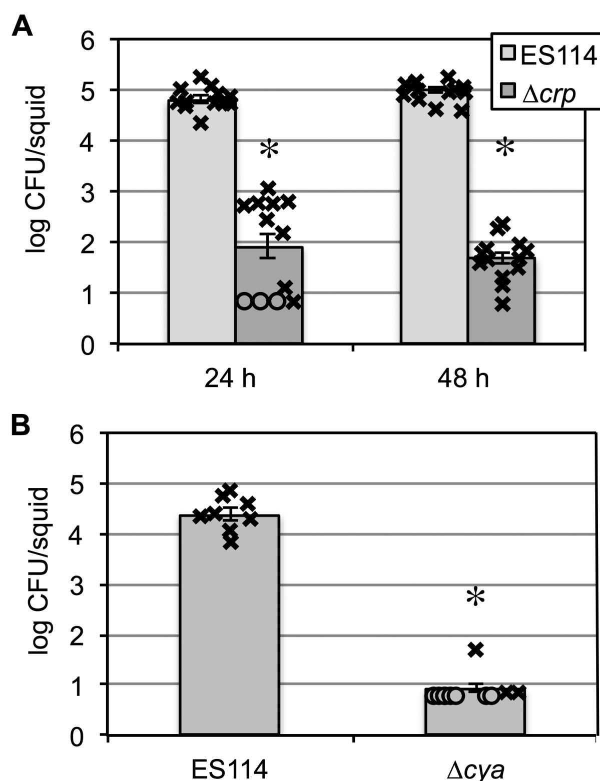

Fig. 1. Symbiotic colonization of aposymbiotic *E. scolopes* juveniles.
A. Colonization by ES114 or JB24 (Δ*crp*) 24 and 48 h post inoculation; ES114 inoculum, 1700 CFU ml^{-1}; Δ*crp* inoculum, 6500 CFU ml^{-1} ($n = 12–13$).
B. Colonization by ES114 or DC03 (Δ*cyaA*) 48 h post inoculation; ES114 inoculum, 2800 CFU ml^{-1}; Δ*cyaA* inoculum, 2000 CFU ml^{-1} ($n = 10–11$). Asterisks indicate significant difference between ES114 and mutant ($P < 0.05$). Average colonization is presented with bars indicating standard error. X's indicate the *V. fischeri* CFU from an individual squid, and O's indicate individual squid with no CFU, plotted at the limit of detection.

To place the symbiotic importance of *cyaA* and *crp* in perspective, we also evaluated growth of Δ*crp* and Δ*cyaA* mutants in culture with different carbon sources. We compared the growth rate (gen hr^{-1} at OD_{595} between 0.3 and 0.6) and the growth yield of the these mutants to their wild-type parent ES114, both in a complex base medium (SWTO) and in a defined medium (FMM), each amended with different carbon sources. The Δ*cyaA* and Δ*crp* mutants paralleled each other, growing poorly relative to wild type on all the carbon sources tested, with the exception that amendment with cAMP reversed these defects for the Δ*cyaA* mutant but not for the Δ*crp* mutant (Table S1). Other than when the Δ*cyaA* mutant was amended with cAMP, growth rates of the mutants were less than half that achieved by ES114 (Table S1). Glucose and NAG supported substantially better growth rates for the Δ*cyaA* and Δ*crp* mutants relative to growth in glycerol or mannose. Moreover, on glucose, NAG or fructose, the Δ*crp* and Δ*cyaA* mutants eventually reached growth yields that were equivalent to, or even higher than, ES114 (Table S1). By contrast, when media were supplemented with mannose, cellobiose or glycerol, the mutants' growth yields remained under half that of ES114. Our data suggest that the Δ*crp* and Δ*cyaA* mutants are attenuated under most growth conditions; however, these growth defects in culture are not nearly as severe as the colonization defects.

To investigate cAMP-CRP-mediated expression in wild-type symbionts, we developed a *gfp* transcriptional reporter under the control of a cAMP-CRP-dependent promoter ($P_{CRP\text{-}D}$). We designed a cAMP-CRP-dependent promoter with a CRP binding site overlapping a relatively weak –35 element, along with a strong –10 site and randomized 'N' bases in between (Fig. 2A). Although based loosely on the *E. coli lac* promoter (a Class II CRP-dependent promoter), this scheme eliminated non-CRP regulatory sites like the *lac* operator. Variants of this template (e.g. Fig. 2B) were cloned upstream of *gfp* in pJLS27 (Fig. 2C), which also encodes a constitutive mCherry gene (Boles and Horswill, 2008). Clones were screened in DC55 (Δ*cpdP* Δ*cpdA* Δ*cyaA*) for responsiveness to exogenous cAMP. By screening several clones, we identified pDC85, in which the *gfp* reporter has relatively low background but a high dose-dependent response to cAMP (Fig. 2D). Expression of this reporter was also dependent on *crp* (see below). Juvenile squid were then infected with ES114 carrying pDC85 or pJLS27. Compared with aposymbiotic squid or squid infected by ES114 carrying the promoterless *gfp*, the $P_{CRP\text{-}D}$-*gfp* reporter showed strongly elevated green fluorescence, indicating cAMP-CRP activity is induced in symbionts at some point during the infection and colonization of the light organ in the 48 h after inoculation (Fig. 3A). This reporter likewise showed activity in symbionts during the first 24 h (data not shown).

Fig. 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 but 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. 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 dose-dependent response of pDC85 in strain DC55 (Δ*cpdP* Δ*cpdA* Δ*cyaA*) to added cAMP.

cAMP-CRP is less active in cells growing on glucose

We also observed the pDC85 reporter in *V. fischeri* strains grown on media supplemented with different carbon sources (Fig. 3B). To test whether $P_{CRP\text{-}D}$-*gfp* is dependent on CRP, we moved pDC85 into the Δ*crp* mutant and examined colonies grown on media supplemented with glucose, NAG, glycerol or mannose. Our results confirmed that the $P_{CRP\text{-}D}$-*gfp* reporter is not active in the absence of *crp*, as indicated by the red fluorescence of

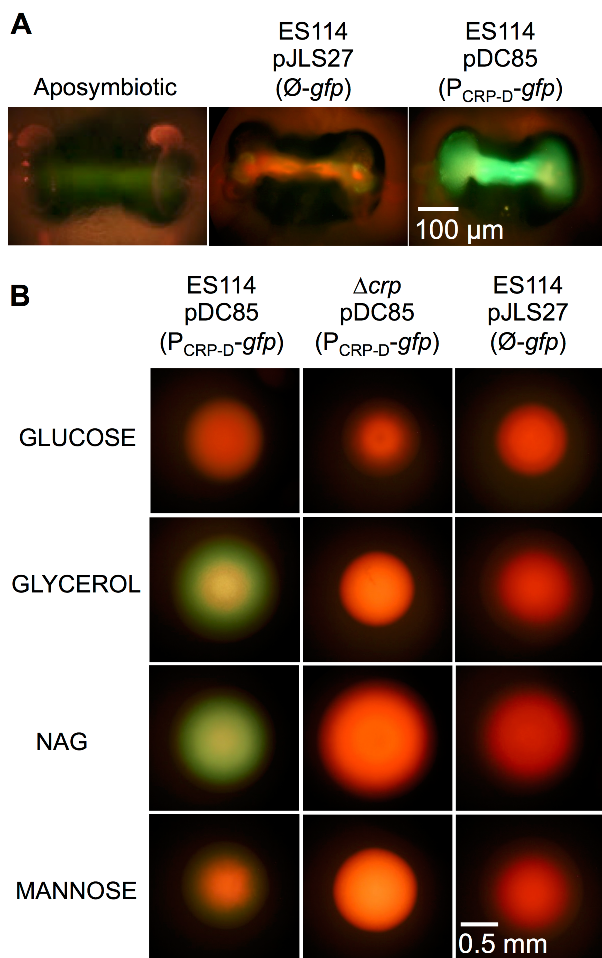

Fig. 3. Epifluorescence images of *E. scolopes* light-organ and *V. fischeri* colonies.
A. Light organs from squid that were un-colonized or 'apo-symbiotic', colonized by ES114 with promoterless-*gfp* (Ø-*gfp*) vector pJLS27, or colonized by ES114 with pDC85 ($P_{CRP\text{-}D}$-*gfp*) 48 h post inoculation.
B. *V. fischeri* colonies grown on SWTO agar supplemented with indicated carbon source. $P_{CRP\text{-}D}$ in ES114 (left) and Δ*crp* (middle) compared with promoterless parent vector pJLS27 in ES114 (right).

the constitutive mCherry and lack of green fluorescence in Δ*crp* mutant JB24 (Fig. 3B). Similar results were seen with the promoterless parent vector in ES114 (Fig. 3B), confirming that green fluorescence is dependent on the expression of the artificial $P_{CRP\text{-}D}$ promoter. We predicted that cAMP-CRP activity, reflected in expression of GFP from the reporter, would be low during growth on glucose but induced in colonies growing on glycerol, which proved to be the case (Fig. 3B). Colonies growing on medium supplemented with NAG were similar to those grown on glycerol, whereas those grown with mannose gave somewhat intermediate results (Fig. 3B). Thus, consistent with its role in other bacteria, cAMP-CRP shows higher activity on non-glucose carbon sources.

Intracellular cAMP levels are higher in cells grown on glucose

Given the control of cAMP-CRP in *E. coli* and other Proteobacteria, 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. 4A). This surprising result was reproducible and statistically significant ($P < 0.05$). We estimate that the intracellular cAMP concentrations in ES114 grown under these conditions were approximately 15–45 µM, which falls within a range that would be physiologically relevant in *E. coli* (Pastan and Perlman, 1970; Peterkofsky and Gazdar, 1974). Figure 4A reports cAMP levels for cells grown in minimal FMM medium at 0.4 OD_{595}; however, we similarly observed higher cAMP levels in glucose-supplemented cells relative to glycerol-supplemented cells at OD_{595} of 0.2 and 0.6 (Fig. 4B). At each of these culture densities (OD_{595} of 0.2, 0.4 or 0.6), under the same growth conditions as our cAMP measurements, the cAMP-CRP dependent reporter showed higher GFP expression when the medium was supplemented with glycerol as opposed to glucose (Fig. 5), as we saw in complex rich medium supplemented with glycerol or glucose (Fig. 3B).

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

We next 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

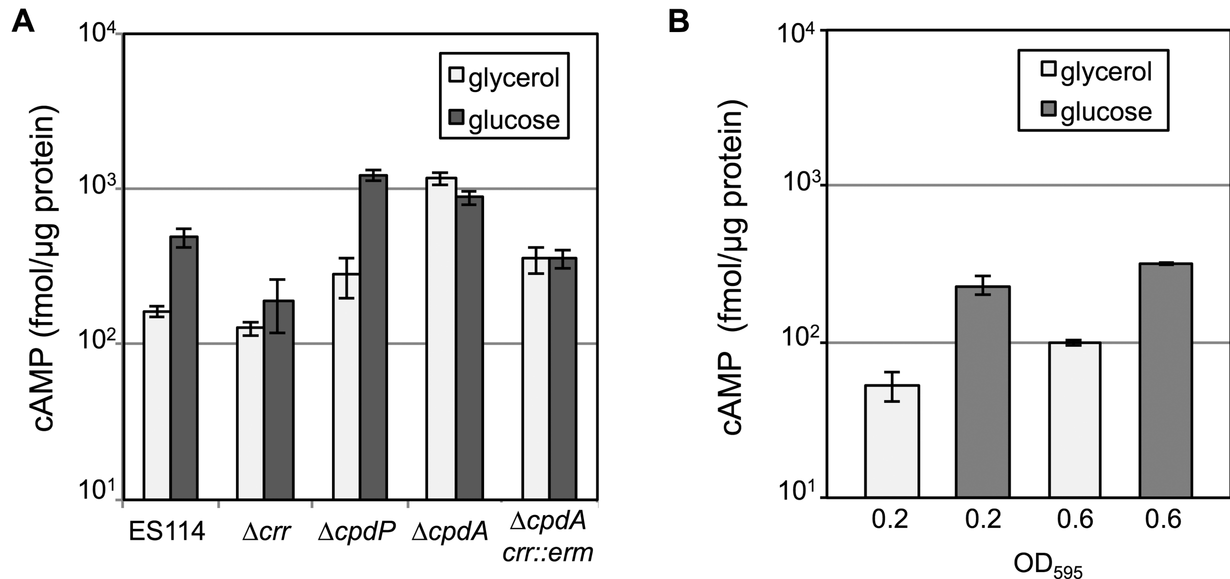

Fig. 4. Intracellular cAMP for *V. fischeri* strains.
A. Cells were grown to OD_{595} of 0.4 in FMM amended with 40 mM glycerol or 20 mM glucose. cAMP in cell lysate was normalized to µg of protein. Error bars represent standard error. Data from multiple experiments were combined with ES114 in each experiment giving similar values. We estimate that 100 fmol cAMP μg^{-1} protein on this scale is equivalent to an intracellular concentration of ~ 10 µM cAMP.
B. intracellular cAMP measured for ES114 grown to OD_{595} 0.2 or 0.6.

phosphodiesterase (Dunlap and Callahan, 1993; Callahan *et al.*, 1995), and deleting *cpdP* resulted in ~ 2-fold higher intracellular cAMP compared with ES114 (Fig. 4). Moreover, much like the wild type, the Δ*cpdP* mutant displayed higher intracellular cAMP when grown on glucose relative to glycerol (Fig. 4). A more likely candidate for influencing intracellular cAMP levels appeared to be CpdA, which

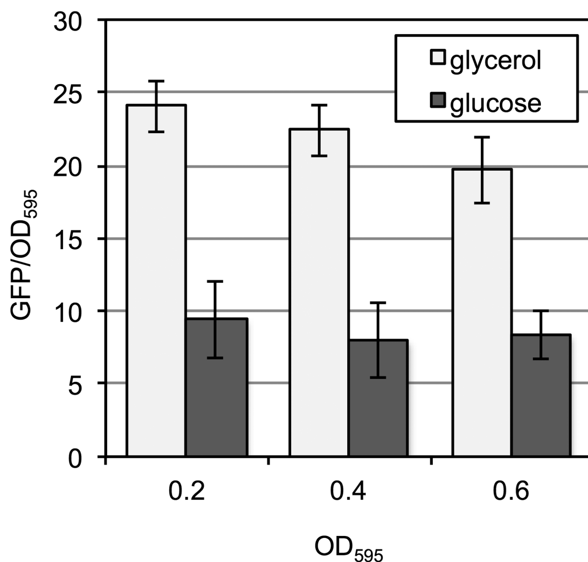

Fig. 5. GFP expression from ES114 pDC85 (P_{CRP-D}-*gfp*). Cells were grown to OD_{595} of 0.2, 0.4 or 0.6 in FMM amended with 40 mM glycerol or 20 mM glucose. Error bars represent standard deviation ($n = 4$).

resembled cytoplasmic cAMP phosphodiesterases from *E. coli* (Imamura *et al.*, 1996) and *Vibrio vulnificus* (Kim *et al.*, 2009), 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. 4), consistent with a 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. 4).

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 in *E. coli*. We saw no significant difference in cAMP levels between ES114 and the Δ*crr* mutant in glycerol-grown cells ($P > 0.05$); however, when a Δ*cpdA* mutant was compared with a Δ*cpdA crr*- double mutant, a significant ($P < 0.05$) drop in intracellular cAMP was seen (Fig. 4). 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 (Saier *et al.*, 1975), and in *E. coli* extracellular cAMP can con-

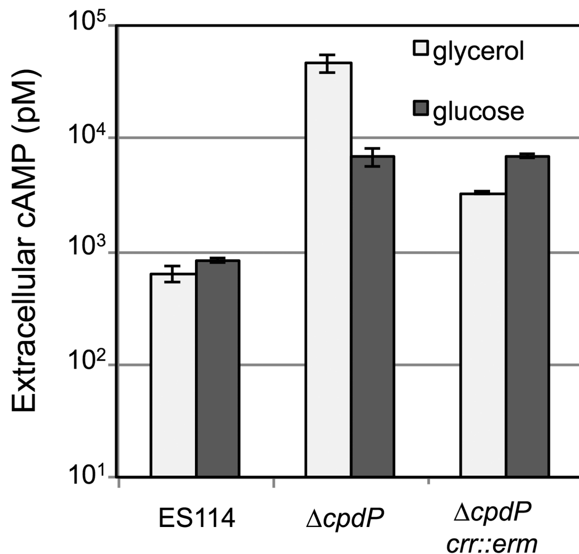

Fig. 6. Extracellular cAMP in *V. fischeri* cultures. Strains were grown to OD_{595} of 0.4 in FMM amended with 40 mM glycerol or 20 mM glucose. Error bars represent standard error. Data from multiple experiments were combined with ES114 in each experiment giving similar values.

stitute ~ 90% of the total cAMP in culture (Peterkofsky and Gazdar, 1974; Hantke *et al.*, 2011). We therefore tested whether differential cAMP export could account for changes in intracellular cAMP. Wild-type cells grown on glycerol or glucose showed no difference in extracellular cAMP ($P > 0.05$) (Fig. 6); however, the periplasmic cAMP phosphodiesterase CpdP might consume extracellular cAMP and obscure differences in cAMP export. In the Δ*cpdP* mutant, we observed 10- to 100-fold more extracellular cAMP than for wild type grown on glucose or glycerol respectively (Fig. 6). Additionally, extracellular cAMP was sevenfold higher in glycerol-grown cells as opposed to glucose (Fig. 6). 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 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. Although the difference was not clearly significant ($P = 0.059$), we found that in glycerol-grown cells, the combined intracellular and extracellular cAMP was twofold higher than in glucose-grown cells (Fig. 7). 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}.

The effect of glucose on cAMP-CRP activity can be independent of cAMP

Given the 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 assess cAMP-CRP activity, we placed the light-generating *luxCDABEG* genes on the *V. fischeri* chromosome under control of the artificial cAMP-CRP-dependent promoter (P_{CRP-D}) described above (Figs 2 and 3). As controls, we generated strains where *luxCDABEG* was either promoterless or under control of an artificial IPTG-inducible promoter. We also generated a strain with a mutant *crp* allele encoding a CRP that acts independently of cAMP. By placing this mutant allele in a Δ*cyaA* mutant background, we enabled CRP-dependent gene regulation but eliminated controls over cAMP levels. We obtained an *E. coli* cAMP-independent *crp** allele with amino acid changes T127L/S128I (Youn *et al.*, 2006) that we exchanged onto the chromosome of a Δ*cyaA* mutant of *V. fischeri* at the *crp* locus. Unexpectedly, recombination occurred between the *E. coli crp** sequence and the *V. fischeri crp* gene, rather than the downstream flanking sequence, resulting in a chimeric *crp* that encoded the first 63 amino acids of *V. fischeri* CRP, and the remaining

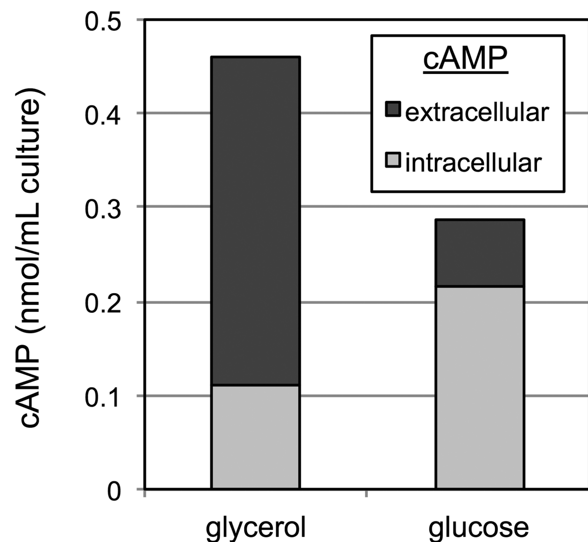

Fig. 7. Comparison of intracellular cAMP to extracellular cAMP. Both intracellular and extracellular cAMP was measured from cultures of DC48 (Δ*cpdP* Δ*cpdA*) grown in FMM amended with 40 mM glycerol or 20 mM glucose to an OD_{595} of 0.4.

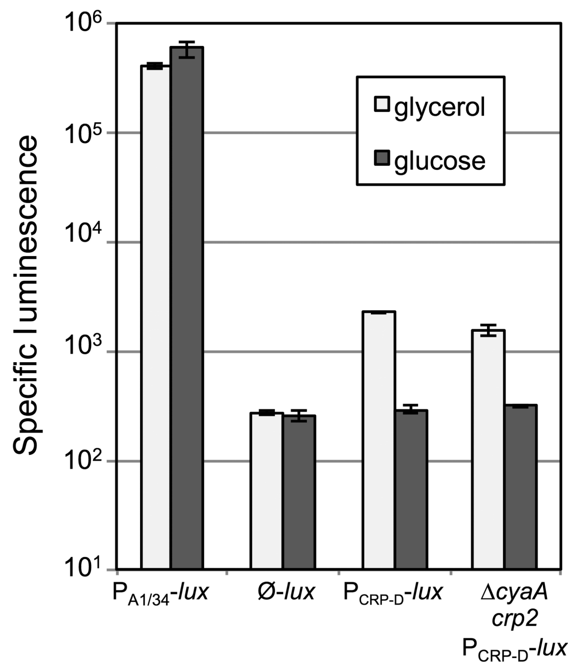

Fig. 8. Expression of CRP-dependent reporter in wild-type *crp* and cAMP-independent *crp2* backgrounds. Specific luminescence was determined for strains including DC57 (P_{CRP-D}-*lux*), DC63 (Δ*cyaA crp2* P_{CRP-D}-*lux*) and JB22 ($P_{A1/34}$-*lux*) grown to OD_{595} of 1.0 on SWTO amended with 40 mM glycerol or 20 mM glucose. Background luminescence is represented by DC08, which carries a promoterless-*lux* (Ø-*lux*) allele. Error bars represent standard error ($n = 2$).

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 the *crp* allele 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 previously shown to make constitutively active CRP variants (Kim *et al.*, 1992). We used this T127L/S128I/G141S CRP* variant (*crp2*) below.

Glucose caused decreased expression of the chromosomal P_{CRP-D}-*lux* reporter (Fig. 8), in much the same way that it affected a plasmid-borne P_{CRP-D}-*gfp* reporter (Figs 3 and 5). Indeed, in media supplemented with glucose, the P_{CRP-D}-*lux* reporter showed no higher expression than a promoterless ($P_{Ø}$-*lux*) control (Fig. 8). A luminescent control with *lux* expressed from an IPTG-inducible promoter ($P_{A1/34}$-*lux*) was not significantly affected by glucose, indicating that the observed effects are dependent on the promoter and CRP-dependent regulation. Finally, we observed that even in a Δ*cyaA crp2* background, where CRP* activity is decoupled from cAMP levels (and cAMP is absent), glucose still decreased CRP activity (Fig. 8).

Glucose can affect cAMP-CRP activity independently of native CRP transcription

To explore whether transcriptional regulation of *crp* is necessary for the glucose-mediated effect on cAMP-CRP activity, *V. fischeri crp* was cloned downstream of a constitutive non-native promoter on a plasmid and moved into a Δ*crp* mutant of *V. fischeri* with the P_{CRP-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. 9). These data were similar to the 10-fold difference we saw between glycerol- and glucose-grown cells with the P_{CRP-D}-*lux* reporter strains with *crp* under native regulation on the chromosome (Fig. 9). Thus, this glucose-mediated effect on cAMP-CRP activity is not dependent on native *crp* transcription.

Discussion

In this study, we investigated control of cAMP-CRP activity in *V. fischeri* as well as the importance of this regulator during symbiotic colonization of the *E. scolopes* light organ. Using a fluorescent cAMP-CRP-dependent transcriptional reporter (Fig. 2), we found that cAMP-CRP is active in symbiotic cells (Fig. 3), and both Δ*crp* and Δ*cyaA* mutants displayed very poor colonization of *E. scolopes* (Fig. 1). The reason(s) for this symbiotic attenuation may be multifactorial, reflecting the broad cAMP-CRP regulon. Among the known cAMP-CRP-controlled genes in *V. fischeri* are *luxR* and *ainSR* (Lyell *et al.*, 2013), which are

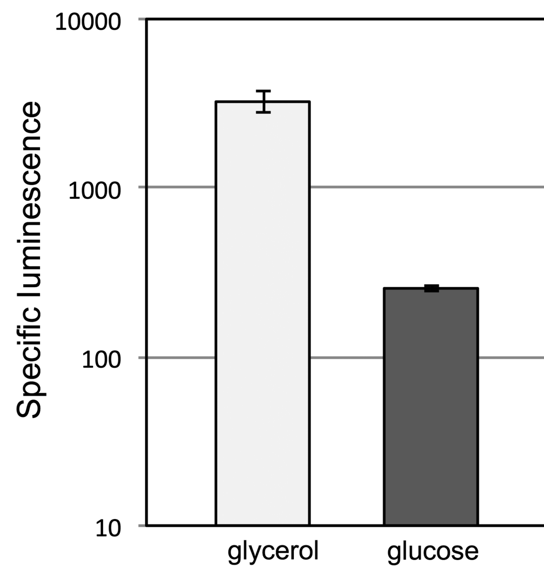

Fig. 9. Expression of CRP-dependent reporter with transcriptionally constitutive *crp*. Specific luminescence was determined for DC68 (Δ*crp* P_{CRP-D}-*lux*) with plasmid-expressed *crp* (pJLB146) grown to OD_{595} of 1.0 on SWTO amended with 40 mM glycerol or 20 mM glucose. Error bars represent standard error ($n = 2$).

involved in pheromone-mediated signaling and bioluminescence (Stabb *et al*., 2008). The Δ*crp* (Lyell *et al*., 2013) and Δ*cyaA* mutants are dim, and luminescence is a colonization factor (Visick *et al*., 2000; Bose *et al*., 2008; Koch *et al*., 2014); however, weak luminescence alone cannot account for the colonization defect of the Δ*crp* and Δ*cyaA* mutants. A completely dark Δ*luxCDABEG* mutant is only attenuated three- to fourfold 48 h after infection (Bose *et al*., 2008), whereas the Δ*crp* and Δ*cyaA* mutants were far more attenuated, close to 1000-fold, at the same time point (Fig 1). The LuxR and AinS/AinR regulons extend beyond luminescence (Lupp and Ruby, 2005; Antunes *et al*., 2007; Qin *et al*., 2007; Studer *et al*., 2008; Cao *et al*., 2012), but the symbiotic defects of *luxR* and *ainS* mutants are also not as severe as those of the Δ*crp* and Δ*cyaA* mutants (Visick *et al*., 2000; Lupp and Ruby, 2004).

One might argue that the poor colonization of the Δ*crp* and Δ*cyaA* mutants reflects a generalized attenuation 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 S1); however, growth rates and growth yields of the mutants in culture (Table S1) are not as starkly different from wild type as are their symbiotic defects. Growth yields in particular were similar for the mutants and wild type under several culture conditions, and growth rates were relatively less compromised when the cells were provided glucose or NAG. It is tempting to speculate that neither glucose, NAG nor cAMP are major carbon sources for *V. fischeri* as it initially colonizes 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, which also would account for our data. Further investigation of the CRP regulon in *V. fischeri* may reveal other important factors in its symbiotic lifestyle.

Because our cAMP-CRP-dependent $P_{CRP\text{-}D}$-*gfp* reporter was used with wild-type symbionts, the results are more straightforward to interpret and do not invoke concerns regarding the arguably generalized attenuation of *crp* and *cyaA* mutants. As noted above, these data indicate that cAMP-CRP is active in symbionts, and they are also consistent with the idea that glucose is not a major carbon source available to symbionts (Fig. 3). This synthetic fluorescent reporter should be useful in further studies regarding the cellular control of cAMP-CRP, as it enabled us to rapidly assess cAMP-CRP activity under a variety of growth conditions, with some unanticipated results, including the intermediate response to growth on mannose (Fig. 3B), that need to be reconciled in future models. Likewise, given the high degree of CRP conservation among the Proteobacteria, the $P_{CRP\text{-}D}$-*gfp* reporter should be useful in several other bacteria and allow researchers to assess the nutritional status of different symbionts in their hosts.

We also investigated if and how CRP activity is affected in response to carbon sources, glucose in particular, in *V. fischeri*. Several lines of evidence suggested that CRP plays 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. A Virtual Footprint analysis (Munch *et al*., 2005) of the CRP regulon in *V. fischeri* suggests potential CRP binding sites upstream from many genes and operons involved in catabolism of non-glucose carbon sources, and in some instances experiments corroborated these bioinformatic predictions. For example, there is a predicted CRP binding site upstream from the cellobiose (*cel*) utilization operon, and glucose leads to decreased expression from the *cel* promoter (Adin *et al*., 2008). Our data in this study further support a classical role for CRP. As predicted, Δ*crp* and Δ*cya* mutants of *V. fischeri* grew much better when supplemented with glucose (Table S1), and more direct confirmation was provided by CRP-dependent reporters, which showed lower activity in glucose-grown cells (Figs 3, 5 and 8). 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 in *E. coli*; however, the relative importance of these mechanisms, and the importance of cAMP levels themselves, were different in *V. fischeri* from what has been reported in *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 twofold increase in intracellular cAMP, attributed to a low K_m for cAMP (Imamura *et al*., 1996), whereas a *V. fischeri* Δ*cpdA* mutant had 10-fold higher cAMP levels (Fig. 3). Moreover, as discussed below, the effect of the $EIIA^{Glc}$ transport component on cAMP levels, which may be important in *E. coli* (Levy *et al*., 1990), 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 with that of *E. coli*.

In *E. coli*, cAMP levels are modulated in response to glucose when adenylate cyclase is activated by the phosphorylated form of the $EIIA^{Glc}$ component of the glucose-transport system, which is encoded by *crr* (Saier and Roseman, 1976; Bettenbrock *et al*., 2007). 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 or Δ*cpdP* mutant backgrounds in which cAMP levels were elevated (Figs 3 and 4). 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. 4A). However, we speculate that cAMP export is the major determinant underlying this difference in intracellular cAMP. 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 the major mechanisms of cAMP turnover, the total cAMP appeared to be twofold higher with glycerol than with glucose (Fig. 7), although the difference was on the border of statistical significance ($P = 0.059$). Thus, our data are at least 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 (e.g. Fig. 7) highlight the importance of understanding cAMP export. An outer membrane channel protein, TolC, has been proposed to be involved in cAMP export in *E. coli* (Hantke *et al.*, 2011; Li and Young, 2014), although those studies have relied primarily on enhanced responsiveness of *tolC* mutants to exogenous cAMP rather than direct measurements of intracellular or extracellular cAMP. Given what is known, it seems possible that TolC might play a role in membrane permeability to exogenous cAMP, rather than cAMP export, in *E. coli*. Our preliminary experiments with a Δ*tolC* mutant of *V. fischeri* yielded no evidence for a role in cAMP export (data not shown). Similarly, a preliminary screen for cAMP export-deficient transposon-insertion mutants was unsuccessful (see *Supporting information*), although future attempts using the cAMP-CRP-dependent reporter as a screening tool might be more fruitful. Understanding the mechanisms of cAMP export should be a research priority, as it remains arguably the least understood aspect of cAMP homeostasis in bacteria but could play a major role in determining cAMP levels.

Our data raise new questions about the role of CpdP. Differences in extracellular cAMP concentration were only evident in *V. fischeri* mutants lacking CpdP, a periplasmic phosphodiesterase unusual in its specificity for 3′-5′-cyclic nucleotides. Upon discovering CpdP, Dunlap *et al.* speculated that it may have a role in degrading free cAMP in the seawater or during symbiotic infection (Callahan *et al.*, 1995). In light of our findings, perhaps 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 interorganismal 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 reportedly 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.

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 (Fig. 8). Under some conditions, CRP protein levels are lower in *E. coli* during growth on glucose (Ishizuka *et al.*, 1993). Although we did not quantify CRP, our initial studies did not reveal such a stark effect on CRP concentration as that reported by Ishizuka *et al.* (1993) (Fig. S1). Native transcriptional regulation of *crp* similarly did not appear to be required for the effect of glucose on cAMP-CRP activity (Fig. 9).

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. CRP can be acetylated (Kuhn *et al.*, 2014), although it remains 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 (Wang *et al.*, 2010). Interestingly, Castano-Cerezo *et al.* provided evidence that cAMP-CRP may regulate the tran-

Table 1. Select 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*	Hanahan (1983)
DH5αλ*pir*	DH5α lysogenized with λ*pir*	Dunn *et al.* (2005)
CC118λ*pir*	Δ(*ara-leu*) *araD Δlac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	Stabb and Ruby (2002)
V. fischeri		
DC03	ES114 Δ*cyaA*	This study
DC08	ES114 promoterless-*lux*	This study
DC18	ES114 Δ*cpdP*	This study
DC25[b]	ES114 Δ*cyaA crp1*	This study
DC26[c]	ES114 Δ*cyaA crp2*	This study
DC38	ES114 Δ*cpdA*	This study
DC42	ES114 Δ*cpdA crr::ermR*	This study
DC48	ES114 Δ*cpdP* Δ*cpdA*	This study
DC51	ES114 Δ*cpdP crr::ermR*	This study
DC55	ES114 Δ*cpdP* Δ*cpdA* Δ*cyaA*	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*	Boettcher and Ruby (1990)
JB22	ES114 $P_{AI/34}$-*luxCDABEG*	Bose *et al.* (2008)
JB24	ES114 Δ*crp*	Bose *et al.* (2007)
JAS202	ES114 Δ*crr*	J. Schwartzman
Plasmids[d]		
pCRP	*E. coli crp** (CRP* T127L/S128I)	Youn *et al.* (2006)
pDC5b	Δ*cyaA* allele; ColE1, *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*, *ermR*	This study
pDC16	*luxR-luxI*; three transcriptional terminators; promoterless-*luxC*	This study
pDC40	Δ*cpdP* allele; ColE1, *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*, *camR*	This study
pDC41	Δ*crp* allele Δ*NheI*; ColE1, *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*, *camR*	This study
pDC42	*E. coli crp** allele; ColE1, *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*, *camR*, *ampR*	This study
pDC54	Δ*cpdA* allele; ColE1, *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*, *camR*	This study
pDC82	*luxR-luxI*; transcriptional terminators; P_{CRP-D}-*luxC*	This study
pDC85	*ori*VR6Kγ, *oriT*$_{RP4}$, pES213, *mCherry, kanR*, P_{CRP-D}-*camR-gfp*	This study
pEVS104	conjugative helper plasmid; *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*	Stabb and Ruby (2002)
pJLB117	Δ*crp* allele; ColE1, *ori*VR6Kγ, *oriT*$_{RP4}$, *kanR*, *camR*	Bose *et al.* (2007)
pJLB146	*crp* in shuttle vector pVSV105; pES213, *ori*VR6Kγ, *camR*	Bose *et al.* (2007)
pJLS27	*ori*VR6Kγ, *oriT*$_{RP4}$, pES213, *mCherry, kanR*, promoterless-*camR-gfp*	This study
pTMO152	*crr::ermR*; *ori*VR6Kγ	Visick *et al.* (2007)
pVSV105	*ori*VR6Kγ, *oriT*$_{RP4}$, pES213, *camR*	Dunn *et al.* (2006)

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, making an 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 (Dunn *et al.*, 2006).

scription of an *E. coli* acetyltransferase, YfiQ (or PatZ), which is involved in regulating acetate metabolism (Castano-Cerezo *et al.*, 2011). 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 on CRP activity was still seen in a *yfiQ* mutant (data not shown). Future work should focus on analysis of *V. fischeri*'s CRP to test for acetylation or other post-translational modifications of this protein in cells grown on glycerol or glucose.

The effects broadly known as catabolite repression, and more narrowly the effects of cAMP-CRP, are understood to extend beyond a response specifically to glucose mediated by $EIIA^{Glc}$ and cAMP levels (Magasanik, 1961; Ishizuka *et al.*, 1993; You *et al.*, 2013). Nonetheless, this study is an important illustration of how much yet remains to be understood about this important global regulatory process in bacteria.

Experimental procedures

Strains, growth media and reagents

Select strains and plasmids used in this study are listed in Table 1. *V. fischeri* ES114 was the wild-type strain used (Boettcher and Ruby, 1990). *E. coli* strains DH5α (Hanahan, 1983) or DH5αλpir (Dunn *et al.*, 2005) were used for cloning,

with the latter used to maintain plasmids with the R6K origin of replication. *E. coli* cultures were grown at 37°C in Luria–Bertani (LB) medium (Miller, 1992) with 20 μg ml^{-1} chloramphenicol, 40 μg ml^{-1} kanamycin or 60 μg ml^{-1} ampicillin when appropriate for selection. *V. fischeri* was grown at 28°C in LB Salt (LBS) medium (Stabb *et al*., 2001), at 24°C in Seawater Tryptone Marine-Osmolarity (SWTO) medium (Bose *et al*., 2007) or at 24°C in supplemented fischeri minimal medium (FMM) (Septer *et al*., 2011) 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. When appropriate for selection of *V. fischeri*, 2 μg ml^{-1} chloramphenicol, 40 μg ml^{-1} kanamycin or 5 μg ml^{-1} erythromycin were added to LBS. cAMP was obtained from Sigma-Aldrich (St Louis, MO).

Plasmids, oligonucleotides, molecular techniques and sequence analyses

Plasmids and mutants were constructed using standard techniques as previously described (Bose *et al*., 2008; Kimbrough and Stabb, 2013). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table S2. 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 (Stabb and Ruby, 2002) in strain CC118λ*pir* (Herrero *et al*., 1990), and allelic exchange was confirmed by PCR. Plasmid construction is described in greater detail in Appendix 2 of the Supporting Information.

Determining growth rate and growth yield

To assess growth rate and yield for *V. fischeri*, strains were grown in either SWTO or FMM amended with glucose, glycerol, NAG, mannose or cellobiose. In some cultures, 5 mM cAMP was added along with glycerol or glucose. Overnight cultures were diluted 1:1000 or 1:100 and incubated at 24°C with shaking at 200 r.p.m. Within each experiment, flask and dilution conditions were uniform. Culture density was determined by measuring OD_{595} at 30 min or 1 h time points in a Biophotometer (Brinkman Instruments, Westbury, NY) and doubling time (gen hr^{-1}) was calculated using data between OD_{595} 0.3 and 0.6. The data reported are representative of multiple independent experiments. Growth yield was determined by the final OD_{595} reached for these cultures, and the reported values are averages of three independent experiments. When OD_{595} exceeded 3.0, which was the maximum value read by the Biophotometer, cultures were diluted 1:10 in medium, remeasured and OD_{595} was calculated based on the dilution factor.

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 r.p.m. 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 s, and relative luminescence was measured immediately with a Glomax TD-20/20 luminometer (Promega, Madison, WI) (Bose *et al*., 2008). 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 −80°C 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 Bio-Rad (Hercules, CA).

Another kit, DetectX Direct cyclic AMP Enzyme Immunoassay (Arbor Assays, Ann Arbor, MI) was used to assay intracellular and extracellular cAMP for DC48 (Fig. 7). In these experiments, 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.

Squid colonization assays and microscopy

To assess squid colonization, *V. fischeri* strains were grown in ASWT at 28°C without shaking to an OD_{595} between 0.4 and 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 h and then moved to inoculum-free Instant Ocean. After 24 or 48 h, 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 (Nikon, Melville NY, USA). 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 (Nikon, Melville NY, USA) filter set was used to visualize the constitutive red fluorescence along with the green fluorescent reporter simultaneously.

Acknowledgements

We thank Ned Ruby and Julia Schwartzman for providing JAS202, Alex Horswill for providing pAH9, Hiroji Aiba for providing anti-CRP antisera and Jorge Escalante-Semerena and Tim Hoover for helpful discussion. The National Science Foundation supported this research under grants OCE-0929081 and IOS-1121106.

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