Published in final edited form as:
FEBS Lett. 2008 November 12; 582(27): 3744–3750. doi:10.1016/j.febslet.2008.10.008.

Cyclic AMP post-transcriptionally regulates the biosynthesis of a major bacterial autoinducer to modulate the cell density required to activate quorum sensing

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Abstract

In *Vibrio cholerae*, expression of the quorum sensing regulator HapR is induced by the accumulation of a major autoinducer synthesized by the activity of CqsA. Here we show that the cAMP-cAMP receptor protein complex regulates *cqsA* expression at the post-transcriptional level. This conclusion is supported by the analysis of *cqsA-lacZ* fusions, the ectopic expression of *cqsA* in Δ*crp* mutants and by Northern blot analysis showing that *cqsA* mRNA is unstable in Δ*crp* and Δ*cya* (adenylate cyclase) mutants. Addition of cAMP to the culture of a Δ*cya* mutant restored *cqsA* mRNA stability and CAI-1 production. Lowering intracellular cAMP levels by addition of D-glucose increased the cell density required to activate HapR. These results indicate that cAMP acts as a quorum modulator.

Keywords

Vibrio cholerae; signal transduction; quorum sensing; cAMP; cAMP receptor protein; carbon catabolite repression

1. Introduction

Vibrio cholerae is a Gram-negative highly motile bacterium that colonizes the human small intestine and produce cholera toxin (CT) which causes the profuse watery diarrhea typical of cholera [1,2]. The expression of CT is regulated by quorum sensing. Quorum sensing is a process by which bacterial cells communicate with one another by secreting extracellular signaling molecules termed autoinducers. Two autoinducer systems function in *V. cholerae*. The cholera autoinducer 1 (CAI-1) synthase CqsA is responsible for the biosynthesis of CAI-1 [(S)-3-hydroxytridecan-4-one] which at high cell density binds to its receptor CqsS [3]. Autoinducer 2 (AI-2) is a furanosyl borate diester [(2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate] synthesized by the activity of LuxS [4] and recognized by receptor LuxPQ. Accumulation of CAI-1 and AI-2 activates the expression of the master

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quorum sensing regulator HapR [5]. HapR plays a pivotal role in regulating virulence gene expression, protease production and biofilm formation [6-8] by modulating intracellular cyclic diguanylate (c-di-GMP) levels, as well as by acting at specific promoters that control virulence gene expression and exopolysaccharide biosynthesis [9,10].

The cAMP receptor protein (CRP) is a global regulator well known for its role in carbon catabolite repression: the inhibition of gene expression by the presence of a rapidly metabolizable carbon source (i.e. glucose) in the growth medium [11-13]. In carbon catabolite repression, the presence of glucose indirectly inhibits adenylate cyclase through a component of the posphoenolpyruvate-dependent phosphotransferase system (PTS) leading to low intracellular cAMP levels [14]. Conversely, when glucose is limited (or replaced by a poor carbon source), the activity of adenylate cyclase increases leading to high intracellular levels of cAMP and formation of the cAMP-CRP complex [12]. The cAMP-CRP complex binds as a dimer to the consensus sequence TGTGA-(N_6)-TCACA which can be found within, adjacent or upstream of responsive promoters [11-13].

In a previous study we demonstrated that CRP is required for expression of the CAI-1 synthase CqsA [15]. Consequently, deletion of *crp* was shown to impact the expression of HapR, multiple HapR-regulated genes and phenotypes such as production of hemagglutinin (HA)/protease, CT, exopolysaccharide biosynthesis and biofilm formation [15,16]. In this study we show that cAMP levels post-transcriptionally activate the expression of CqsA by enhancing the stability of the *cqsA* mRNA. Thus, cAMP acts upstream of quorum sensing to regulate CAI-1 biosynthesis and modulate the cell density required to activate HapR. Finally, this is the first report of the cAMP-CRP complex modulating gene expression at the level of mRNA stability to integrate carbon catabolite repression and quorum sensing.

2. Materials and methods

2.1. Strains and media

V. cholerae mutants used in this study were constructed from the El Tor biotype strain C7258 (Perú isolate, 1991). Construction of strains AJB61 (C7258Δ*cqsA*), WL7258 (C7258Δ*crp*), C7258Δ*lacZ* and WL7258Δ*lacZ* have been described previously [15,17]. *E. coli* TOP10 (Invitrogen) and S17−1λ*pir* [18] were used for cloning purposes. *V. cholerae* strains were grown in Bacto tryptic soy broth (TSB) (Becton, Dickinson & Co.) or LB at 37°C with agitation (250 rpm). When necessary, culture media were supplemented with ampicillin (Amp, 100-μg/ml), tetracycline (Tet, 5-μg/ml), kanamycin (Km, 25-μg/ml), X-gal (20-μg/ml) or polymyxin B (100-units/ml). Plasmid DNA was introduced in *V. cholerae* by electroporation [19].

2.2 Construction of mutants

Strain WL7259 (C7258Δ*cya*) containing a deletion of *cya* encoding adenylate cyclase was constructed by transferring the suicide vector pCVDΔ*cya*-km [16] to C7258 followed by sucrose selection as described previously [16]. To construct the Δ*crp*Δ*luxO* double mutant SZS013, chromosomal DNA fragments flanking the *luxO* open reading frame (ORF) were amplified from C7258 genomic DNA with the Advantage 2 PCR kit (Clontech) using the primer sets 5′-GCGCGAGCTCGTGATTTACGATTAGGCG/5′-GCGCGGATCCAAACATCACACATCTAGAC and 5′-GCGCGGATCCGTGAACTCAATGATTAC/5′-GCGCGTCGACAGATAACCTTTCGGTGC and the amplicons were sequentially cloned in pCVD442 [20]. The resulting suicide vector containing the chromosomal *luxO* deletion was transferred to strain WL7258Δ*lacZ* by conjugation and the Δ*luxO* mutant was obtained by sucrose selection as described previously [15,16].

2.3. Construction of plasmids and chromosomally integrated *lacZ* fusions

A 1.6-kb DNA fragment encompassing the *cqsA* ORF flanked by 5′ and 3′ non-coding (intergenic) sequences was amplified using primers 5′-GGGGATCCTGACCGTGATGTATTGCTA and 5′-GAACTGCAGCGCTCAGTAAACTCCTAA and cloned as a *BamH*I-*Sph*I fragment in pBR322 to yield pBRCqsA2383. To construct a *cqsA-lacZ* transcriptional fusion we sequentially cloned the *rrnB*T_1T_2 transcription terminator [21] and a 214-bp fragment containing the *cqsA* promoter region amplified with primers 5′-GGCCAAGCTTTCGCAATATATCCTAGTT and 5′-GAACTGCAGCGCTCAGTAAACTCCTAA in pUC19 to generate pTTcqsA. A 1-kb internal fragment of the C7258 *lacZ* gene ($lacZ^{VC}$) was amplified using primers 5′-CGAAGGTACCAATCCCCGATTCA and 5′-GCCTCTAGATCGCCACCGTTTTACACTG and cloned as a *Kpn*I-*Xba*I fragment in pTTcqsA upstream of the *rrnB*T_1T_2 transcription terminator to generate pZTC. Next, a 1.6-kb *Kpn*I-*Hind*III fragment containing the $lacZ^{VC}$ fragment, the *rrnB*T_1T_2 terminator and the *cqsA* promoter was sub-cloned upstream of the promoterless *E. coli lacZ* gene ($lacZ^{EC}$) in plasmid pKRZ1 [22] to yield pWLZTC. Finally, a 5.6-kb *Pst*I-*Kpn*I DNA fragment containing the $lacZ^{VC}$-*rrnB*T_1T_2 - *cqsA* - $lacZ^{EC}$ unit was cloned as a *Pst*I-*Kpn*I fragment in the suicide vector pCVD442 [20] to yield pCVDZTC. The suicide vector pCVDZTC containing a *cqsA-*$lacZ^{EC}$ transcriptional fusion was mobilized from *E. coli* S17−1λ*pir* to C7258 and WL7258 by conjugation to generate strains C72ZTC and WL72ZTC. Integration of pCVDZTC by homologous recombination results in disruption of *V. cholerae* chromosomal *lacZ* gene. Correct integration was confirmed by PCR and DNA sequencing. To construct *cqsA-lacZ* translational fusions, primer pairs 5′-CGGAATTCCGAGTCTACGACAATGAT/5′-CGGAATTCTATCGCTATCTATTTCGTC and 5′-CCGAATTCCCAGATTGAGATAATAGACA/5′-CGGAATTCTATCGCTATCTATTTCGTC were used to amplify DNA fragments starting within the upstream locus VCA0524 and containing different length of *cqsA* coding sequence, respectively. The PCR products, of size 1183- and 984-bp, were confirmed by DNA sequencing and cloned as *Xba*I-*EcoR*I fragments in the suicide vector pVIK111 [23] to generate the translational *cqsA-lacZ* (in-frame) fusions contained in plasmids pVIK1648 and pVIK1847, respectively. Plasmid pVIK1648 contains DNA encoding the first 174 amino acid of CqsA while pVIK1847 contains DNA encoding the first 107 amino acids. Plasmids pVIK1648 and pVIK1847 were transferred by conjugation from S17−1λ*pir* to C7258Δ*lacZ* and WL7258Δ*lacZ.* Stable integration within the *cqsA* locus was confirmed by PCR and DNA sequencing. To express *cqsA* from a heterologous Tac promoter, primers 5′-CGGGATCCGATGAACAAGCCTCAACT and 5′-GCTCTAGATGACCGTGATGTATTGCT were used to amplify the *cqsA* ORF and 3’-UTR. The PCR product was confirmed by DNA sequencing and cloned as a *BamH*I-*Pst*I fragment in pALTER-Ex2 (Promega) to create pTac-CqsA. Construction of plasmid pHapRLac2 containing a *hapR-lacZ* fusion has been described previously [24].

2.4. RNA techniques

For total RNA preparation, cells were treated with the RNA stabilizing reagent RNA Protect (QIAGEN Inc.) and total RNA was isolated using the RNeasy kit and RNase-free DNAse set (QIAGEN Inc.). Total RNA was fractionated in a 1% agarose - 20 mM MOPS - 5 mM sodium acetate - 2 mM EDTA - 2% formaldehyde gel and transferred to positively charged nylon membranes (Roche Applied Sciences) by capillary transfer. The membranes were hybridized to a *cqsA* probe in DIG Easy Hyb (Roche) at 50°C overnight, washed and developed with an alkaline phosphatase-conjugated anti-digoxigenin antibody and CSDP (Roche). To determine the stability of *cqsA* in different genetic backgrounds, cells were grown in TSB to a predetermined cell density, treated with rifampicin (200-μg/ml) to block transcription and

samples were taken subsequently at different time points for Northern blot analysis. The primer pairs 5′-CGGGATCCCTAGGATATATTGCGATG/5′-GCTCTAGATGACCGTGATGTATTGCT and 5′-GTGCTGTGGATGTCATCGTTGTTG/5′-CGCTTTACCTTGGCCGATTT were used to generate DIG-labeled *cqsA* and *recA* DNA probes, respectively. To this end, the resulting PCR products were gel-purified and labeled by random priming using the DIG-random prime kit (Roche).

2.5 Measurement of β-galactosidase and protease activities

β-Galactosidase activity was measured as described by Miller [25] using the substrate o-nitrophenyl-β-D-galctotopyranoside (ONPG). Specific activities are given in Miller units [1000 (OD_{420} / t .v .OD_{600})] where t is reaction time and v is the volume of enzyme extract per reaction. Expression of HA/protease [26] was measured using an azocasein assay as described previously [27]. One azocasein unit is the amount of enzyme producing an increase of 0.01 OD_{442} units per h.

2.6. Assay of CAI-1 activity

To measure the production of CAI-1 activity, cultures were centrifuged at 12,000 rpm for 10-min and the supernatants were filtered through a 0.22-μm syringe filter. Cell-free culture supernatants were tested for the presence of CAI-1 activity by inducing light production in the *V. cholerae* reporter strain MM920 containing the cosmid pBB1, which carries the *V. harveyi lux* operon [5]. The reporter strain was grown overnight with shaking at 30°C, diluted 1:10 in fresh medium, and 70-μL aliquots transferred to an opaque-wall 96-well microtiter plate. Cell-free culture fluids were added to a final concentration of 30 % (v/v). The plates were incubated at 30°C with agitation and light production was measured at 30-min intervals in a Genios Plus Tecan luminometer. Results are expressed as light fold induction relative to a sterile medium control.

3. Results

In a previous study we showed that production of CAI-1 requires an active *crp* allele [15]. According to common knowledge, the activity of *E. coli* CRP is determined by the intracellular concentration of cAMP [14]. However, genetic variants of CRP have been identified exhibiting significant cAMP-independent activity [28]. Contrary to *E. coli*, the *V. cholerae* CRP protein has not been extensively characterized. To determine if the strong *cqsA* dependency on CRP can be fully accounted by cAMP binding to CRP, we constructed an adenylate cyclase deletion mutant. In Fig. 1 we show that no CAI-1 could be detected in strain WL7259 lacking adenylate cyclase. The CAI-1 defect of strain WL7259 could be complemented by introducing the wild type (WT) *cya* gene on plasmid pTTCya [16] (Fig. 1, shadowed bar). Furthermore, production of CAI-1 in strain WL7259 could be restored by supplementing the medium with 2.5- or 5.0-mM cAMP. As expected, addition of cAMP to the medium did not restore CAI-1 expression in strain WL7258 lacking CRP (Fig. 1). These results indicate that expression of CqsA is under positive regulation by the cAMP-CRP complex (Fig. 1). Since CRP is a global regulator which affects the expression of many genes [15], we found it important to confirm that CRP regulation of CAI-1 is the major mechanism by which CRP modulates HapR. CAI-1 acts to inactivate phospho-LuxO, a repressor of HapR [5]. We have shown that Δ*crp* mutants do not make HapR-dependent HA/protease [15,27]. As expected, deletion of *luxO* from strain WL7258Δ*lacZ* (Δ*crp*) to generate SZS013 (Δ*crp*Δ*luxO*) fully restored HA/protease production (azocasein units/OD_{600}: C7258Δ*lacZ*, 18.2 ± 0.4; WL758Δ*lacZ*, 0.9 ± 0.4; SZS013, 24.3 ± 1.6) suggesting that CRP regulation of CAI-1 is the major regulatory event by which CRP controls the quorum sensing master regulator HapR. We used the software virtual footprint ((http://www.prodoric.de/vfp/) to scan the 5′ un-translated DNA preceding *cqsA* for putative

cAMP-CRP binding sites. This analysis did not return high-scoring putative binding sites suggesting that CRP regulates *cqsA* indirectly and/or post-transcriptionally.

To determine if cAMP-CRP regulation of *cqsA* is at the level of transcription or post-transcriptional, we constructed strains harboring chromosomally integrated transcriptional and translational *cqsA-lacZ* fusions. In the transcriptional fusions, *cqsA* 5′ un-translated DNA was ligated to a promoterless *E. coli lacZ* gene in plasmid pCVDZTC and recombined into the chromosome of strains C7258 (WT) and WL7258 (Δ*crp*). No significant differences in β-galactosidase expression were detected between the WT and the Δ*crp* mutant suggesting that the regulation of *cqsA* by cAMP-CRP does not involve the 5′ un-translated DNA preceding the *cqsA* ORF (Fig. 2A). Next, we constructed two chromosomally integrated translational (in-frame) fusions containing DNA encoding the first 107 (pVIK1847) and 174 (pVIK1648) amino acids of CqsA ligated to a *lacZ* gene devoid of translation initiation signals. As shown in Fig. 2B, the expression of both translational fusions was strongly CRP-dependent indicating that the *cqsA* region responsive to CRP resides within the DNA encoding the first 107 N-terminal amino acids of CqsA. To further confirm that regulation of *cqsA* by CRP is post-transcriptional, we tested whether ectopic expression of *cqsA* using a heterologous promoter was still CRP-dependent. To this end, we constructed pTac-CqsA expressing *cqsA* from the strong Tac promoter. The Tac promoter lacks the CRP responsive region of the WT *lac* promoter and is not sensitive to cAMP [29]. In addition, *V. cholerae* does not produce a *lac* repressor resulting in constitutive expression of genes placed under the control of this promoter. In Fig. 2C we show that pTac-CqsA effectively complemented a Δ*cqsA* (*crp*-positive) mutant. However, expression of *cqsA* from pTac-CqsA was still significantly diminished in the isogenic Δ*crp* mutant (Fig. 2C). Taken together, the above results strongly suggest that cAMP-CRP post-transcriptionally regulates the expression of CqsA.

The level of *cqsA* mRNA produced in a Δ*crp* mutant is below the level of detection of the chemiluminescence detection system described in methods. To determine the size of the *cqsA* transcript produced in the Δ*crp* mutant, we decided to boost the amount of *cqsA* mRNA by placing the *cqsA* gene on plasmid pBRCqsA2383. The plasmid was introduced by electroporation in Δ*cqsA* and Δ*crp* deletion mutants and the resulting transformants were analyzed for production of CAI-1. Although pBRCqsA2383 fully complemented the Δ*cqsA* mutant for CAI-1 production (fold Induction > 1000), very little CAI-1 (fold induction < 50) was detected in the Δ*crp* mutant suggesting that placing the *cqsA* gene on a multicopy plasmid can not titrate off its requirement for the cAMP-CRP complex. Production of *cqsA* mRNA by these transformants was investigated by Northern blot analysis. In the WT strain (C7258), a single *cqsA* mRNA band which migrated below a 1517-bp marker could be detected (Fig. 3, **lane 1**) suggesting that the 1170-bp *cqsA* ORF is not co-transcribed with other genes. A ribonuclease protection assay indicated that the *cqsA* mRNA produced in C7258 starts approximately 39-bp upstream the *cqsA* translational start (data not shown). Introduction of pBRCqsA2383 in strain AJB61 (Δ*cqsA*) resulted in over expression of the *cqsA* full length transcript (Fig. 3, **lane 3**). However, very little amount of full length transcript could be detected in the Δ*crp* mutant (Fig. 3, **lane 2**). Instead, a diffuse material of smaller molecular weight hybridizing to the *cqsA* probe was detected (Fig. 3, **lane 2, arrow**). This result suggests that a low level of a modified (shorter) *cqsA* transcript might be produced in the Δ*crp* mutant either by *de novo* synthesis or degradation of the full length transcript.

We decided to analyze the longevity of the *cqsA* mRNA in WT, Δ*crp* and Δ*cya* genetic backgrounds using non-steady state Northern blot analysis. To visualize sufficient full length *cqsA* transcript for this analysis, we introduced pBRCqsA2383 in strain AJB61 (Δ*cqsA*) and WL7258 (Δ*crp*). The transformants were grown in TSB to OD_{600} 1.0 and rifampicin was added to block transcription. Next, samples were taken at different time points to determine *cqsA* mRNA decay. As shown in Fig. 4A, no *cqsA* mRNA could be detected 5-min after rifampicin

addition in the Δ*crp* mutant. In contrast, an identical experiment using the complemented strain AJB61 (Δ*cqsA*) showed that the *cqsA* mRNA could be detected 20-min after the addition of rifampicin (Fig. 4A). Since addition of cAMP to TSB medium restored the ability of an adenylate cyclase mutant to produce CAI-1 (Fig. 1), we examined if exogenous cAMP acted by stabilizing the *cqsA* mRNA. To this end, strain WL7259 (Δ*cya*) was grown in TSB to OD_{600} 0.5, the culture was divided in halves and 5-mM cAMP was added to one half (the other used as a control). One h later, rifampicin was added to block transcription and samples were taken from each culture to assess the longevity of *cqsA* mRNA. As shown in Fig. 4B, no *cqsA* mRNA could be detected in the Δ*cya* mutant either at time zero or 5-min after the addition of rifampicin. Contrastingly, supplementation of the WL7259 culture with 5-mM cAMP resulted in production of a more stable *cqsA* mRNA that could be detected 30-min after the addition of rifampicin (Fig. 4B). The time-dependent decay of *recA* mRNA was used as an independent positive control of the rifampicin-induced transcription block. In summary, the above results strongly suggest that formation of the cAMP-CRP complex positively affects *cqsA* expression at the level of mRNA stability. Thus, inactivation of either *crp* or *cya* leads to the production of an unstable *cqsA* mRNA that does not sustain CqsA expression and production of CAI-1. Based on the finding that cAMP controls the rate of CAI-1 production, we hypothesized that lowering intracellular cAMP levels by supplementation of the culture medium with D-glucose [14,30] should increase the cell density required to activate expression of HapR. To test this hypothesis, we introduced plasmid pHapRLac2 containing a *hapR-lacZ* fusion that displays the typical cell density-dependent (U-shaped) pattern of quorum sensing regulated phenotypes in strain C7258Δ*lacZ* [24]. As shown in Fig. 5, the glucose-supplemented culture required a higher cell density to activate HapR expression.

4. Discussion

Results reported in this study are relevant to how bacteria integrate and respond to multiple sensory information. Here we focus on the transduction of two major external signals: carbon source and population density. Global gene expression profiling of a *V. cholerae* Δ*crp* mutant showed that CRP is required for *V. cholerae* to make CAI-1 [15]. Results shown in Fig. 1 further demonstrate that CRP acts through the conventional cAMP activation mechanism. The significance of this result is that it establishes a novel small molecule signaling pathway by which the primary messenger cAMP activates the production of (S)-3-hydroxytridecan-4-one which functions as an autoinducer to turn on quorum sensing signal and activate HapR. In turn, HapR has been shown to regulate the intracellular concentration of the second messenger c-di-GMP [9]. Therefore, our data suggests the existence of a cAMP – (S)-3-hydroxytridecan-4-one – c-di-GMP signaling pathway modulating a broad range of cellular activities. Our results provide a new paradigm for the integration of carbon catabolite repression and quorum sensing. *V. cholerae* cells resolve integrating carbon source and population density sensory information by placing the biosynthesis of a major autoinducer under cAMP control. Consequently, the intracellular concentration of cAMP acts upstream of quorum sensing to control the major input signal required for cell-to-cell communication in response to carbon source. This interpretation is consistent with previous observations showing that glucose strongly represses the production of CAI-1, *hapR* mRNA and the HapR-dependent hemagglutinin/protease [15,24,27]. In fact, we here demonstrate that a higher cell density is required to activate HapR under conditions that lower intracellular cAMP such as supplementation of the culture medium with D-glucose (Fig. 5). We therefore define cAMP as acting as a quorum modulator. It has been reported that production of AI-2 in *E. coli* [31] and more recently in *Edwardsiella tarda* [32] is modulated by cAMP. Thus, cAMP regulation of the biosynthesis of an autoinducer molecule could be a general strategy by which bacteria integrate population density with other features of the extracellular milieu.

The result of our *cqsA-lacZ* transcriptional and translational fusion analysis is consistent with cAMP-CRP acting at the post-transcriptional level. This conclusion is further sustained by the finding that ectopic expression of *cqsA* from a heterologous cAMP-independent constitutive promoter is still diminished in a Δ*crp* mutant. Moreover, non-steady state Northern blot analysis demonstrated that the *cqsA* mRNA is rapidly degraded in Δ*crp* and Δ*cya* mutants but can be rescued (in the Δ*cya* mutant) by supplementing the medium with pure cAMP. To our knowledge this is the first report of cAMP-CRP controlling gene expression at the level of mRNA stability. It should be recognized that the instability of *cqsA* mRNA in *crp* and *cya* mutants could result from a translation defect. Since CRP is not known to have RNA binding activity, it is likely that it acts by modulating the expression of an unknown protein or small RNA (sRNA) that could interact with *cqsA* mRNA to exert the expected regulation. We have recently observed that *in vitro* transcribed *cqsA* does not cleave itself in the absence of added protein or RNA factors (unpublished results). This observation favors a mechanism in which CRP is required to express a protein or sRNA factor that protects *cqsA* mRNA from catalytic rather than autocatalytic degradation.

We have used the program TargetRNA (http://snowwhite.wellesley.edu/targetRNA/index_2.html) to find potential sRNAs in the *V. cholerae* genome capable of base pairing with the *cqsA* mRNA encoding the CqsA N-terminal region required for CRP regulation. However, none of the hits obtained with this software identified known sRNAs or exhibited additional predictive features of sRNAs. The RNA analyzer software RegRNA (http://regrna.mbc.nctu.edu) revealed two sm-sites ($RAU_{3-6}GR$, R = purine nucleotide) within the *cqsA* N-terminal coding sequence. These sites occur in small nuclear ribonucleoproteins (snRNP) that participate in eukaryotic pre-mRNA splicing. One small bacterial sm-like protein that regulates quorum sensing is Hfq [33]. However, deletion of *hfq* in a Δ*crp* background did not restore *cqsA* expression (unpublished results). Therefore, it is likely that CRP regulation of *cqsA* mRNA stability represents a novel pathway independent of Hfq and known sRNAs. We have used the program RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) to examine the secondary structure of the *cqsA* mRNA region which, according to our deletion and *cqsA-lacZ* fusion data, should contain the cAMP-CRP responsive element. This analysis revealed that the mRNA encoding the N-terminal sequence responsible for cAMP-CRP regulation can adopt a stable secondary structure with several simple and complex multipartite stem loops including the translational start. Analysis of this sequence using riboswitch finder (http://riboswitch.bioapps.biozentrum.uni-wuerzburg.de/server.html) or RNA analyzer (http://rnaanalyzer.bioapps.biozentrum.uni-wuerzburg.de/server.html) did not reveal any known motif. Furthermore, the region of *cqsA* capable of forming a stable secondary structure was analyzed for suboptimal conformations using the vRNAsubopt software of the Vienna RNA package (http://emboss.bioinformatics.nl/cgi-bin/emboss/vrnasubopt). Suboptimal structures calculated with this program differed only slightly and did not affect the *cqsA* translational start. Since cAMP-CRP regulation of *cqsA* mRNA stability and CAI-1 production appears to involve unknown protein and/or sRNA regulators not predictable by current computational approaches, a genetic screening is underway in our laboratory to identify regulatory factors linking cAMP-CRP to *cqsA* mRNA expression.

5. Acknowledgements

This work was supported by PHS Grant RO1AI063187 and 2S06GM008248-20 from the National Institutes of Health to J.A.B. and A.J.S. respectively. We are grateful to the National BioResource Project (NIG, Japan) for providing plasmid pVIK111.

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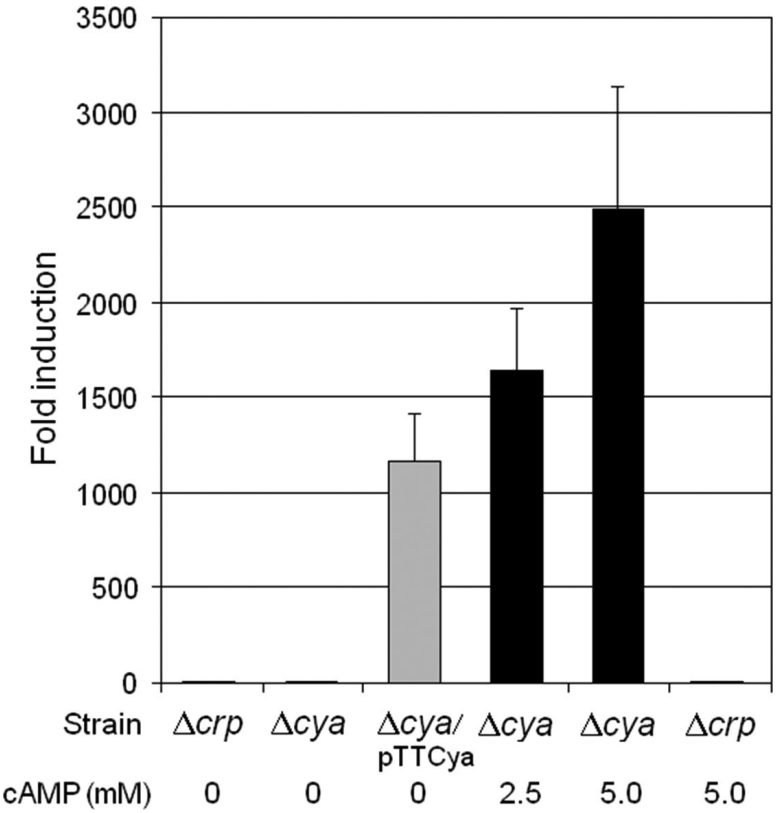

Fig. 1. Exogenous cAMP restores the capacity of an adenylate cyclase mutant to produce CAI-1

Overnight cultures of WL7259 (Δ*cya*) were diluted 100-fold in fresh TSB and grown to OD_{600} 0.5. Each culture was divided in three portions and cAMP (Sigma Chemical Co.) was added to a final concentrations of zero (control), 2.5- and 5-mM respectively. The cultures were incubated at 37°C for 1-h with shaking and the amount of CAI-1 in the cell-free supernatant was determined as described in methods. Strain WL7258 (Δ*crp*) and strain WL7259 (Δ*cya*) containing the complementing plasmid pTTCya (shadowed bar) were used as controls. Each value represents the average of three independent cultures. Error bars indicate the standard deviation.

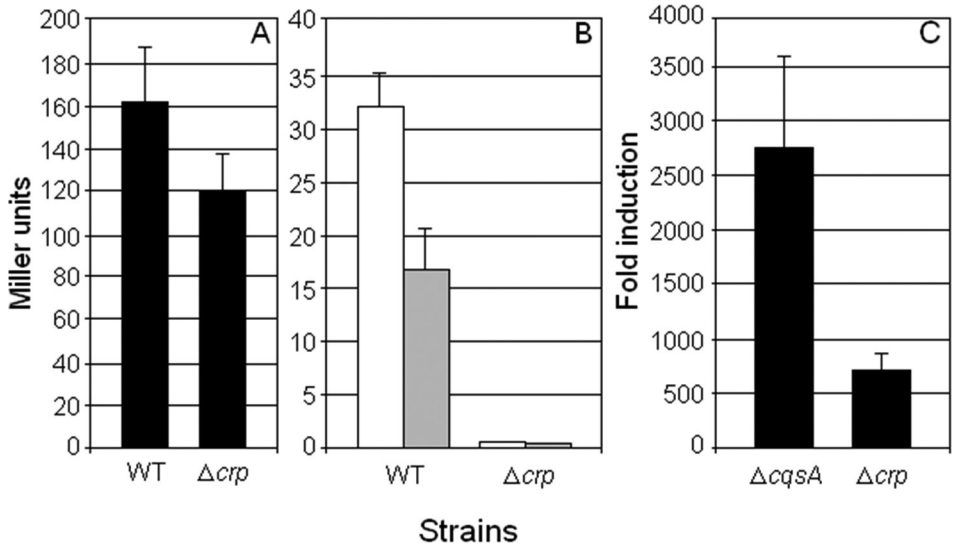

Fig. 2. Gene fusion analysis of cAMP-CRP regulation of *cqsA* expression

(A). Transcriptional fusion. β-Galactosidase expression in strains C72ZTC (WT background) and WL72ZTC (Δ*crp* background) containing a *cqsA-lacZ* transcriptional fusion. Strains were grown at 37°C with agitation in LB overnight. **(B). Translational fusions**. β-Galactosidase expression in strains C7258Δ*lacZ* (WT background) and WL7258Δ*lacZ* (Δ*crp* background) containing the *cqsA-lacZ* translational fusions pVIK1847 (open bar) or pVIK1648 (shadowed bar). Strains were grown at 37°C with agitation in LB overnight. **(C). Ectopic expression of *cqsA***. Strains AJB61 (C7258Δ*cqsA*) and WL7258 (C7258Δ*crp*) containing pTac-CqsA were grown at 37°C with agitation in TSB overnight and production of CAI-1 activity was determined as described in methods. In all cases, each value represents the average of three independent experiments. Error bars indicate standard deviations.

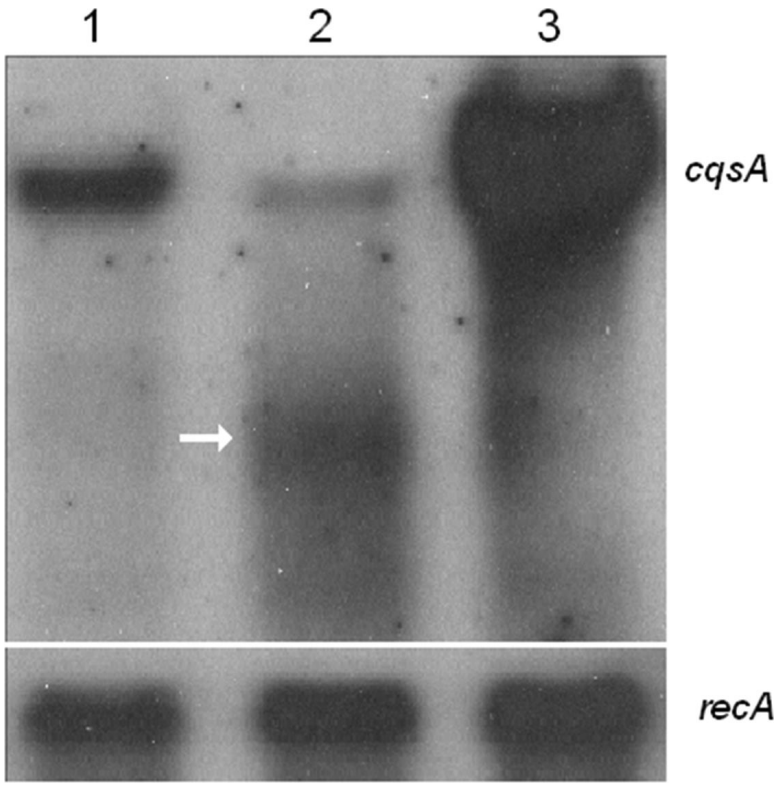

Fig. 3. Analysis of *cqsA* transcription in Δ*cqsA* and Δ*crp* mutants containing plasmid pBRCqsA2383

Samples containing 20-μg of RNA were subjected to Northern blot analysis using a DIG-labeled *cqsA* probe. The *recA* mRNA was used as loading control. Lane 1, C7258; lane 2, WL7258/pBRCqsA2383; lane3, AJB61/pBRCqsA2383.

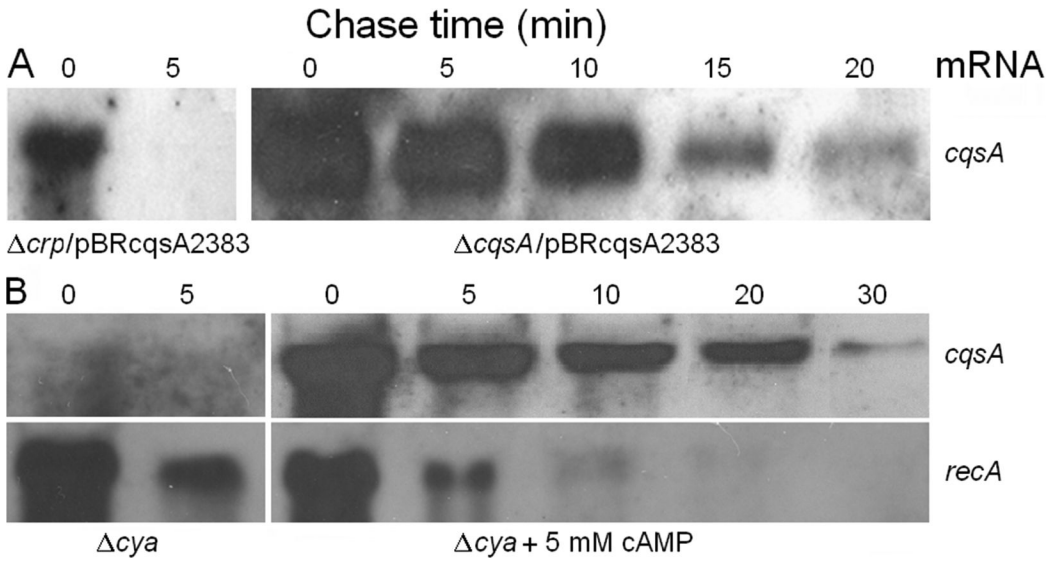

Fig. 4. Longevity of *cqsA* mRNA in WT, Δ*crp* and Δ*cya* genetic backgrounds

(**A**). Overnight cultures of WL7258 (*crp*) and AJB61 (Δ*cqsA*) containing pBRcqsA2383 were diluted 100-fold in fresh TSB and grown at 37°C with agitation to OD_{600} 1.0. Rifampicin was added and samples were taken at the indicated time points for Northern blots using a DIG-labeled *cqsA* probe. Each lane contains 25-μg of total RNA. (**B**). Overnight cultures of WL7259 (C7258Δ*cya*) were diluted 100-fold in fresh TSB and grown at 37°C with agitation to OD_{600} 0.5. Each culture was divided in halves, one half was used as a control and the other half was transferred to a new flask containing cAMP (5-mM). The cultures were incubated 1-h as described above and rifampicin was added to block transcription. Samples were taken at the indicated time points for Northern blot analysis. Each lane was loaded with 25-μg of total RNA. After stripping the *cqsA* probe, the membrane was re-probed with a DIG-labeled *recA* DNA fragment.

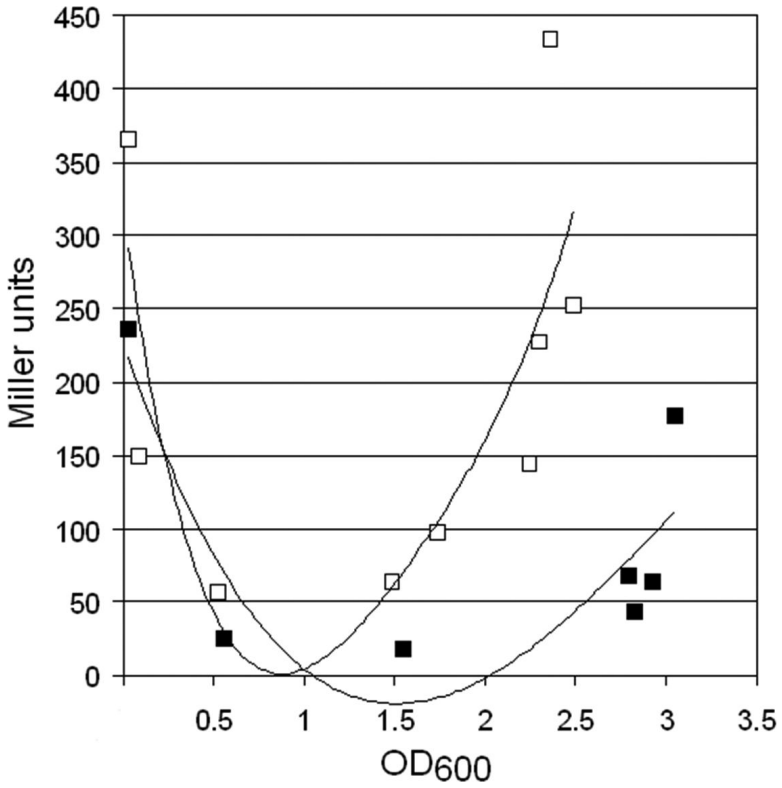

Fig.5. Carbon catabolite repression regulates the cell density or quorum required to activate HapR

Strain C7258Δ*lacZ* containing plasmid pHapRLac2 was grown overnight in LB at 37°C. On the next morning the culture was diluted in fresh LB (□) or LB containing 0.2% glucose (■) and samples taken at different times to determine the expression of β-galactosidase activity. Each point is the average of three independent experiments.