The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*

Weili Liang,¹ Alberto Pascual-Montano,² Anisia J. Silva¹ and Jorge A. Benitez¹

Correspondence

Anisia J. Silva

asilva-benitez@msm.edu

¹Morehouse School of Medicine, Department of Microbiology, Biochemistry and Immunology, 720 Westview Dr. SW, Atlanta, GA, USA

²Computer Architecture Department, Facultad de Ciencias Físicas, Universidad Complutense de Madrid, Madrid 28040, Spain

Vibrio cholerae is the causative agent of cholera, which continues to be a major public health concern in Asia, Africa and Latin America. The bacterium can persist outside the human host and alternates between planktonic and biofilm community lifestyles. Transition between the different lifestyles is mediated by multiple signal transduction pathways including quorum sensing. Expression of the Zn-metalloprotease haemagglutinin (HA)/protease is subject to a dual regulation which involves the quorum-sensing regulator HapR and the cAMP receptor protein. In a previous study, we observed that a mutant defective in the cAMP-receptor protein (CRP) expressed lower levels of HapR. To further investigate the role of CRP in modulating HapR and other signal transduction pathways, we performed global gene expression profiling of a Δcrp mutant of El Tor biotype *V. cholerae*. Here we show that CRP is required for the biosynthesis of cholera autoinducer 1 (CAI-1) and affects the expression of multiple HapR-regulated genes. As expected, the Δcrp mutant produced more cholera toxin and enhanced biofilm. Expression of flagellar genes, reported to be affected in $\Delta hapR$ mutants, was diminished in the Δcrp mutant. However, an epistasis analysis indicated that cAMP–CRP affects motility by a mechanism independent of HapR. Inactivation of *crp* inhibited the expression of multiple genes reported to be strongly induced *in vivo* and to affect the ability of *V. cholerae* to colonize the small intestine and cause disease. These genes included *ompU*, *ompT* and *ompW* encoding outer-membrane proteins, the alternative sigma factor σ^E required for intestinal colonization, and genes involved in anaerobic energy metabolism. Our results indicate that CRP plays a crucial role in the *V. cholerae* life cycle by affecting quorum sensing and multiple genes required for survival of *V. cholerae* in the human host and the environment.

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INTRODUCTION

Vibrio cholerae, the causative agent of Asiatic cholera, produces a Zn-metalloprotease known as haemagglutinin (HA)/protease. This protease, encoded by *hapA* (Häse & Finkelstein, 1991), enhances enterotoxicity in rabbit ileal loops (Silva *et al.*, 2006) and contributes to reactogenicity

Abbreviations: AFC, average fold change; AI-2, autoinducer 2; CAI-1, cholera autoinducer 1; CRP, cAMP receptor protein; CT, cholera toxin; FDR, false discovery rate; HA, haemagglutinin; OMP, outer-membrane protein; qRT-PCR, quantitative real-time PCR; TCP, toxin-co-regulated pilus.

The GEO platform accession number for the TIGR *V. cholerae* version 2 microarray used in this study is GLP4353. The GEO series record number for the raw data files is GSE7519.

A complete list of differentially expressed genes in the Δcrp mutant is provided as supplementary data with the online version of this paper.

in volunteers receiving live genetically attenuated cholera vaccines (Benitez *et al.*, 1999). Expression of HA/protease is regulated by quorum sensing (Jobling & Holmes, 1997; Zhu *et al.*, 2002). Quorum sensing is a process by which bacteria communicate with one another by secreting extracellular signalling molecules termed autoinducers. In *V. cholerae*, two autoinducer/sensor systems have been identified. System 1 consists of cholera autoinducer 1 (CAI-1), synthesized by the activity of CqsA, and its cognate receptor CqsS (Miller *et al.*, 2002). System 2 consists of an AI-2 molecule, synthesized by the activity of LuxS, and its cognate receptor LuxPQ (Miller *et al.*, 2002). At low cell density the autokinase domains of CqsS and LuxPQ become phosphorylated and phosphorus is transferred to LuxU and then LuxO (Henke & Bassler 2004; Miller *et al.*, 2002). LuxO-P activates expression of four small regulatory RNAs (sRNAs), Qrr1–4, which in

conjunction with the RNA-binding protein Hfq destabilize *hapR* mRNA (Lenz *et al.*, 2004; Bejerano-Sagie & Xavier, 2007). When the amount of CAI-1 and AI-2 produced by growing bacteria reaches a threshold value, CqsS and LuxPQ switch from kinase to phosphatase. The flow of phosphorus is reversed and LuxO becomes dephosphorylated and inactive (Henke & Bassler, 2004; Lenz *et al.*, 2004; Miller *et al.*, 2002). At this stage (high cell density), HapR and HA/protease are expressed (Zhu *et al.*, 2002). A third two-component system controlling quorum sensing is VarS/VarA, which regulates transcription of the sRNAs CsrB, CsrC and CsrD (Lenz *et al.*, 2005; Bejerano-Sagie & Xavier, 2007). The sRNAs CsrBCD control the activity of the global regulator CsrA (Lenz *et al.*, 2005; Bejerano-Sagie & Xavier, 2007), which enhances the activity of LuxO-P at low cell density. Recently the small nucleoid protein Fis has been reported to enhance degradation of *hapR* mRNA at low cell density (Lenz & Bassler, 2007).

The HapR regulator plays a pivotal role in regulating virulence gene expression and biofilm development (Hammer & Bassler, 2003; Zhu & Mekalanos, 2003). At low cell density the regulator AphA activates expression of TcpP/H (Häse & Mekalanos, 1998; Kovacikova & Skorupski, 2001), which in concert with a second pair of transmembrane regulators (ToxR/S) activates expression of the soluble regulator ToxT (Miller *et al.*, 1989). ToxT acts positively at the *ctxA* and *tcpA* promoters to activate production of cholera toxin (CT) and the toxin co-regulated pilus (TCP) (DiRita *et al.* 1991). At high cell density, HapR represses the transcription of *aphA* to diminish CT and TCP production (Kovacikova & Skorupski, 2002), represses the exopolysaccharide genes involved in biofilm formation (Yildiz *et al.*, 2004) and activates *hapA* (Jobling & Holmes, 1997; Zhu *et al.*, 2002).

Transcription of *hapA* occurs in the stationary phase and requires the cAMP receptor protein (CRP) and RpoS (Benitez *et al.*, 2001; Silva & Benitez, 2004). Although the *hapA* promoter contains a putative cAMP–CRP binding site, we have not observed binding of purified *V. cholerae* cAMP–CRP to the *hapA* promoter (Silva & Benitez, 2004). Instead, we observed that a *crp::Km* mutant produced lower levels of *hapR* and *rpoS* mRNA (Silva & Benitez, 2004). The *V. cholerae* *hapR* promoter does not contain a cAMP–CRP binding site, suggesting that CRP does not act directly on *hapR*.

The CRP global regulator is the determinant of carbon catabolite repression. Carbon catabolite repression can be defined as the inhibition of gene expression and/or protein activity by the presence of a rapidly metabolizable carbon source in the growth medium (Brückner & Titgemeyer, 2002; Kolb *et al.*, 1993; Stülke & Hillen, 1999). This is achieved through activation of adenylate cyclase by a component of the phosphoenolpyruvate-dependent phosphotransferase system (Deutscher *et al.*, 2006). Activation of adenylate cyclase leads to high intracellular levels of cAMP (Ganguly & Greenough, 1975; Kolb *et al.*, 1993)

and formation of the cAMP–CRP complex, which binds to responsive promoters to activate or repress transcription (Kolb *et al.*, 1993). The cAMP–CRP complex binds as a dimer to the consensus sequence TGTGA-(N₆)-TCACAA, which can be found within, adjacent or upstream of responsive promoters (Brückner & Titgemeyer, 2002; Kolb *et al.*, 1993; Stülke & Hillen, 1999). In Gram-negative bacteria, carbon catabolite repression is a major global regulatory mechanism that influences many signal transduction pathways. CRP has been shown to negatively affect the expression of virulence factors, CT and TCP in classical and El Tor biotype strains grown in LB medium (Skorupski & Taylor, 1997). However, *crp* mutants are defective in intestinal colonization. This suggests that CRP positively controls additional factors required for *V. cholerae* survival in the small intestine. Intestinal colonization is a multifactorial process requiring many genes. It has been shown that *V. cholerae* mutants defective in the alternative sigma factors σ^S (RpoS) and σ^E (RpoE), outer-membrane proteins OmpU and OmpT and motility exhibit diminished colonization of the suckling mouse intestine (Merrell *et al.*, 2000; Kovacikova & Skorupski, 2002a; Provenzano & Klose, 2000; Silva *et al.*, 2006).

To better understand the role of *crp* in pathogenesis, we performed global gene expression profiling of a Δcrp *V. cholerae* mutant of the El Tor biotype. Inactivation of *crp* affected production of CAI-1, multiple HapR-regulated genes, motility, the alternative sigma factor RpoE, outer-membrane proteins and genes involved in anaerobic respiration.

METHODS

Strains and media. *Escherichia coli* and *V. cholerae* strains, plasmids and primers used in this work are listed and briefly described in Tables 1 and 2. *E. coli* strains were grown in LB medium at 37 °C with agitation (250 r.p.m.). *V. cholerae* strains were grown in LB medium at 37 °C. For HA/protease production, they were grown in Bacto tryptic soy broth (TSB) at 37 °C, and for CT production, in AKI medium at 30 °C (Iwanaga *et al.*, 1986). Plasmid DNA was introduced into *V. cholerae* by electroporation (Marcus *et al.*, 1990). Culture media were supplemented with ampicillin (Amp, 100 µg ml⁻¹), tetracycline (Tet, 10 µg ml⁻¹), kanamycin (Km, 100 µg ml⁻¹), X-Gal (40 µg ml⁻¹) or polymyxin B (PolB, 100 units ml⁻¹) as required.

Construction of mutants. Deletion mutants were constructed by allelic exchange using strain C7258 as wild-type precursor. *V. cholerae* target sequences were amplified from genomic DNA of strain C7258 using the Advantage PCR system (BD Biosciences Clontech). Chromosomal DNA was extracted using the Qiagen DNeasy tissue kit. All primers were designed based on the DNA sequence of the *V. cholerae* N16961 genome downloaded from the TIGR database (<http://cmr.tigr.org>). Amplification products were directionally cloned in pUC19 or pUC18 using *E. coli* TOP10 as host and confirmed by sequencing both DNA strands with M13 forward and reverse primers. Amplicons and restriction fragments were purified from agarose gels by using the Qiagen QIAquick gel extraction kit.

In all cases, mutants were obtained by cloning a chromosomal DNA fragment containing a deletion of the target gene in pCVD442. The

Table 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
<i>E. coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG thi thr leu tonA lacY supE recA::RP4-2Tc::Mu</i> (λ <i>pir</i> R6K)	Invitrogen
SM10λ <i>pir</i>		Miller & Mekalanos (1988)
<i>V. cholerae</i>		
C7258	Wild-type, El Tor biotype	Peru isolate, 1991
WL7258	C7258, Δ <i>crp</i>	This study
AJB61	C7258, Δ <i>cqsA</i>	This study
M36	C7258, <i>luxS::Km</i>	This study
AJB51	C7258, Δ <i>hapR</i>	This study
WL51	C7258, Δ <i>hapRcrp</i>	This study
AJB50	C7258, Δ <i>rpoS</i>	This study
MM920	C6706str2 Δ <i>cqsA</i> Δ <i>luxQ</i> containing <i>V. harveyi luxCDABE</i> operon in cosmid pBB1(Tet ^R)	Miller <i>et al.</i> (2002)
Plasmids		
pCVD442	Suicide vector containing R6K <i>ori</i> , <i>sacB</i> , Amp ^R	Donnenberg & Kaper (1991)
pBADCRP7	CRP ORF cloned in pBADHisB	Silva & Benitez (2004)
pCRP1	0.9 kb <i>SacI</i> – <i>Bam</i> HI DNA fragment 5' of <i>crp</i> ORF amplified with primers CRP362/CRP1221 in pUC19	This study
pCRP2	0.8 kb <i>Bam</i> HI– <i>Sph</i> I DNA fragment 3' of the <i>crp</i> ORF amplified with primers CRP1784/CRP2595 in pUC19	This study
pΔCRP	<i>Bam</i> HI– <i>Sph</i> I fragments from pCRP2 in pCRP1	This study
pCVDΔCRP	<i>SacI</i> – <i>Sph</i> I Δ <i>crp</i> fragment from pΔCRP in pCVD442	This study
pHapR1	0.7 kb <i>SacI</i> – <i>Bam</i> HI DNA fragment 5' of the <i>hapR</i> ORF amplified with primers HapR35/HapR754 in pUC19	This study
pHapR2	0.7 kb <i>Bam</i> HI– <i>Xba</i> I DNA fragment 3' of <i>hapR</i> ORF amplified with primers HapR1004/HapR1704 in pUC19	This study
pΔHapR	<i>Bam</i> HI– <i>Xba</i> I fragment from pHapR2 in pHapR1	This study
pCVDΔHapR	<i>SacI</i> – <i>Xba</i> I Δ <i>hapR</i> fragment from pΔHapR in pCVD442	This study
pRpoS1	0.9 kb <i>SacI</i> – <i>Bam</i> HI DNA fragment 5' of <i>rpoS</i> ORF amplified with primers RpoS222/RpoS1131 in pUC19	This study
pRpoS2	0.7 kb <i>Bam</i> HI– <i>Xba</i> I DNA fragment 3' of <i>rpoS</i> ORF amplified with primers RpoS1439/RpoS2140 in pUC19	This study
pΔRpoS	<i>Bam</i> HI– <i>Xba</i> I fragment from pRpoS2 in pRpoS1	This study
pCVDΔRpoS2	<i>SacI</i> – <i>Xba</i> I Δ <i>rpoS</i> fragment from pΔRpoS in pCVD442	This study
pCqsA123	0.9 kb <i>SacI</i> – <i>Bam</i> HI DNA fragment 5' of <i>cqsA</i> ORF amplified with primers CqsA69/CqsA973 in pUC19	This study
pCqsA343	0.9 kb <i>Bam</i> HI– <i>Sph</i> I DNA fragment 3' of the <i>cqsA</i> ORF amplified with primers CqsA1811/CqsA2701 in pUC19	This study
pΔCqsA	<i>Bam</i> HI– <i>Sph</i> I fragments from pCqsA343 in pCqsA123	This study
pCVDΔCqsA	<i>SacI</i> – <i>Sph</i> I Δ <i>cqsA</i> fragment from pΔCqsA in pCVD442	This study
pUC18 <i>luxS</i>	1.3 kb <i>Sall</i> – <i>SacI</i> DNA fragment encoding <i>luxS</i> in pUC18	This study
pUC18 <i>luxS</i> -Km	1.2 kb <i>Sall</i> /Klenow Km ^R cassette from pUC4K in <i>Clal</i> /Klenow site of pUC18 <i>luxS</i>	This study
pCVD <i>luxS</i> -Km	2.5 kb <i>luxS::Km</i> allele from pUC18 <i>luxS</i> -Km in pCVD442	This study

resulting vector was transferred by conjugation to the receptor strain and sucrose selection was applied to isolate segregants retaining the mutant allele. Briefly, to construct *crp* mutants WL7258 and WL51 (Table 1), chromosomal DNA flanking the *crp* ORF was amplified using primer pairs CRP362/CRP1221 and CRP1784/CRP2595, respectively. The amplified DNAs were sequentially cloned in pUC19 to generate plasmids pCRP1, pCRP2 and pΔCRP (Table 1). The chromosomal fragment containing the *crp* deletion was transferred from pΔCRP to pCVD442 (Donnenberg & Kaper, 1991) to generate pCVDΔCRP (Table 1). The suicide vector pCVDΔCRP was constructed in *E. coli* SM10λ*pir* and mobilized to strains C7258

and AJB51 (Table 1) by conjugation. Exconjugants were selected in LB medium containing Amp and PolB and streaked on LB agar containing 15% (w/v) sucrose. Sucrose-resistant colonies were tested for Amp sensitivity and deletion of *crp* was confirmed by DNA sequencing using primers CRP873 and CRP2105. A similar strategy was used to construct the Δ*hapR* mutant AJB51, Δ*rpoS* mutant AJB50 and Δ*cqsA* mutant AJB61 (Table 1). Suicide vectors pCVDΔ*hapR*, pCVDΔ*rpoS*2 and pCVDΔ*cqsA* (Table 1) were transferred by conjugation to C7258; the corresponding mutants were obtained by sucrose selection and confirmed by DNA sequencing. Finally, to construct the *luxS* insertion mutant M36, *luxS* was amplified using

Table 2. Primers

Primer	Sequence (5'–3')
CqsA1424	TTGTTACACCAAATGGCTCCTGCG
CqsA1816	CATCTGCCAGCCCAATACGAATGT
CqsA69	GCGAGCTCATGAGTTTGGTTTACC
CqsA973	GGTGGATCCAAACAAAAGCTGCTAAAC
CqsA1811	GAAGGATCCGCAGATGGTTTGTAAATA
CqsA2701	GTTGCATGCGACCTTAGCCACTTAT
CRP362	CGAGCTCGCCGCTACTTACACACCTT
CRP1221	CGGGATCCTTGGATCGGTTTGAGGTT
CRP1784	CGGGATCCTGCCCACGGTAAACCA
CRP2595	ACATGCATGCCCGCTGATTAATGACCATA
CRP873	CAAGATGAGTTTTGCGATGG
CRP2105	GAAGTAAAAGGCTCACCAGA
Fis56	AAGACCAAATCACGCAAAAGCCACT
Fis190	GCGAGTGATTGTCATGATGGTGTC
FlaA40	GCACAACGTTATCTGACCAAAGGC
FlaA291	GAGTTGGTACCGTTCGCCGATTG
FlaC405	CAAGCTGCTCAACGGTACATTG
FlaC592	GCCTTCCGTTATCTGACCAAGGC
HapR35	GAAGACCTCCATACTGTTGCCATA
HapR754	CAAGGATCCCAAAATTCAGCACATC
HapR1004	CCAGGATCCCGAATTGTGTGAGAAA
HapR1704	GGTTCTAGACGAGTCACTGCAAGAT
HapR589	CGCCTCAAAAACGCAAACTACAAC
HapR1046	ATGGAGTAGAAGATGCCGTGGAAC
LuxSF1	GCATGTCGACTGCCGATCTGCGTGTGATTGC
LuxSR1301	GCTAGAGCTCCCACATCCATGTCTCCAAGCT
RecA578	GTGCTGTGGATGTCATCGTTGTTG
RecA863	CCACCCTTCTTCGCTTCTTTGA
RpoE111	TTCCCGGTATGTAAGCAATTCAGG
RpoE345	CGTAATGTTCTCCGGTTCGATAAT
RpoS222	GCAGAGCTCTCCACCTTACACCAT
RpoS1131	CCAGGATCCAACTCTTCACGAACG
RpoS1439	AGCACCATGGTGGATCCGTCAAAC
RpoS2140	ACTCCTCGCCAAGCTCTAGATATG
VpsL607	ACTGGGCAGGTGCAAAATGTCTATA
VpsL775	AGGGGGTATCAAAAATGCTAAACGC

primers LuxSF1 and LuxSR1301. The amplicon was cloned in pUC18 and *luxS* was subsequently inactivated by insertion of a Km cassette from pUC4K (GenBank accession no. X06404). The *luxS::Km* allele was transferred to pCVD442 and the resulting plasmid introduced by conjugation into C7258. The *luxS::Km* mutant M36 was obtained by sucrose selection and confirmed by DNA sequencing.

Differential gene expression analysis. The *V. cholerae* version 2 microarray (GEO platform accession number GLP4353) consisting of 3811 70-mer oligonucleotides representing ORFs from *V. cholerae* strain N16961 was provided by The Institute for Genome Research Pathogen Functional Genomics Resource Center (<http://pfgrc.tigr.org/>). Wild-type strain C7258 and its Δcrp mutant were grown in LB at 37 °C with agitation (250 r.p.m.) to OD₆₀₀ 1.5. Cells were collected by centrifugation at 4 °C and immediately subjected to RNA extraction. Total RNA was extracted using the Trizol Plus RNA purification system (Invitrogen) followed by the RNeasy MinElute Cleanup (Qiagen). The purity and integrity of RNA samples was verified by UV spectrophotometry and denaturing agarose gel electrophoresis. Using 15 µg total RNA per sample, cDNA was

synthesized in the presence of 3-aminoallyl-2'-deoxyuridine 5'-triphosphate by reverse transcription with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Cy-5 fluorescent dye (Amersham Biosciences) was coupled to the reference (wild-type) cDNA; Cy-3 was coupled to the test (mutant) cDNA. Labelled cDNAs were purified using the MiniElute PCR purification kit (Qiagen) and the cDNA probes mixed and applied to the array surface for hybridization overnight at 42 °C for 16 h. The slides were scanned using the Agilent G2565AA Microarray Scanner System and images processed using the Agilent Technologies Scanner Control Software 6.13 and Agilent Feature Extraction Software 8.5.1.1 (Agilent Technologies). For differential expression, we used the false discovery rate (FDR) to decide the cut-off values for significant genes. To calculate FDR, we used the rank product (RP) test statistic (Breitling *et al.*, 2004), which is a non-parametric method to detect differentially regulated genes in replicated experiments. An average fold change (AFC) greater than 1.5 and a FDR threshold lower than 0.05 was considered to define significantly differentially expressed genes. Results reported in this study represent the average of four experiments. Selected genes found to be differentially expressed in the microarray study were confirmed by quantitative real-time RT-PCR (qRT-PCR).

Reverse transcription PCR. *V. cholerae* strains were grown in LB medium to OD₆₀₀ 1.5 and total RNA extracted as described in the preceding section. To validate microarray data, RNA samples were analysed by qRT-PCR using the iScript two-step RT-PCR kit with SYBR Green (Bio-Rad). Relative expression values (*R*) were calculated using the equation $R = 2^{-\Delta(C_{T\text{target}} - \Delta C_{T\text{reference}})}$, where *C_T* is the fractional threshold cycle. The *recA* mRNA was used as reference. The following primer combinations were used: CqsA1424 and CqsA1816 for *cqsA* mRNA; HapR589 and HapR1046 for *hapR* mRNA; Fis56 and Fis190 for *fis* mRNA; FlaA40 and FlaA291 for *flaA* mRNA; FlaC405 and FlaC592 for *flaC* mRNA; RecA578 and RecA863 for *recA* mRNA; RpoE111 and RpoE345 for *rpoE* mRNA; and VpsL607 and VpsL775 for *vpsL* mRNA. A control mixture lacking reverse transcriptase was performed for each reaction to exclude chromosomal DNA contamination.

Assay of CAI-1 activity. Cell-free supernatants of *V. cholerae* cultures were used as the source of autoinducer molecules. To this end, single colonies were inoculated into 5 ml LB and incubated overnight at 37 °C with shaking. The cultures were then diluted 1:100 in fresh LB and grown to the same optical density (OD₆₀₀ 1.5). Cultures were centrifuged at 17400 g for 10 min and the clear supernatants 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 (Table 1). This reporter strain does not produce CAI-1 and does not respond to AI-2. Briefly, reporter strain MM920 was grown overnight with shaking at 30 °C, diluted 1:10 in fresh medium, and 70 µl aliquots were transferred to an opaque-wall 96-well microtitre 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 GeniosPlus plate reader. The data are reported as peak fold light induction compared to a sterile medium control.

Enzymic and immunosorbent assays. The amount of HA/protease secreted to the medium was measured using an azocasein assay (Benitez *et al.*, 2001). One azocasein unit is the amount of enzyme that produces an increase of 0.01 optical density units h⁻¹ in this assay. CT was determined by GM₁ ELISA using a standard curve of pure CT (Sigma) as described previously (Silva *et al.*, 1998).

Biofilm formation. Biofilm formation was measured by the crystal violet staining method and results normalized for growth and

expressed as the A_{570}/OD_{600} ratio (Zhu & Mekalanos, 2003). Strains were grown in 5 ml LB medium for 16 h, diluted 1:50 in fresh medium and transferred to 96-well flat-bottom microtitre plates. The plates were incubated for 24 h at 30 °C for biofilm development.

Motility assay. Swarm agar plates consisted of LB medium containing 0.3% agar. They were inoculated by stabbing with overnight LB cultures grown from single colonies. Plates were incubated for 24 h at 30 °C.

RESULTS

Global gene expression profiling of a Δcrp mutant

To examine the effect of deleting *crp* on pathogenesis, quorum sensing and biofilm formation, we compared the gene expression profile of strain C7258 and its isogenic Δcrp mutant WL7258 using the TIGR version 2 *V. cholerae* microarray. A total of 176 genes, including *crp* itself (VC2614) were found to be differentially expressed in the Δcrp mutant ($AFC > 1.5$, $FDR < 0.05$), representing 4.4% of *V. cholerae* N16961 predicted genes. As expected, apart from conserved hypothetical proteins, the majority of differentially expressed genes fell in the role categories energy metabolism, transport and binding proteins, and cellular processes (Fig. 1). Furthermore, 79% of the differentially expressed genes were downregulated, suggesting that CRP most frequently acts as a positive regulator in *V. cholerae* under the conditions used. In Table 3 we provide a short list of differentially expressed genes emphasizing those regulated by quorum sensing and/or involved in cholera pathogenesis. A complete list of differentially expressed genes is provided as supplementary data with the online version of this paper. The raw data

Fig. 1. Genes differentially expressed in a *V. cholerae* El Tor biotype Δcrp mutant by TIGR role category.

files have been deposited in Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) and assigned series record number GSE7519.

CRP is required for CAI-1 expression and modulates quorum sensing

The *cqsA* gene encoding CAI-1 synthase (VCA0523) was found to be downregulated in the Δcrp mutant (Table 3). In order to validate the microarray results, we performed qRT-PCR. The Δcrp mutant expressed very little *cqsA* mRNA compared to its wild-type precursor in this assay (Fig. 2a). As expected, cell-free supernatants of the Δcrp mutant contained negligible amounts of CAI-1 activity (Fig. 2b). To confirm that CRP is required for CAI-1 production, we performed a complementation test by introducing plasmid pBADCRP7 (Table 1), which expresses *crp* from the arabinose (*araBAD*) promoter. We have shown that this plasmid encodes a biologically active CRP protein that exhibits cAMP-dependent binding to a bona fide cAMP–CRP binding site (Silva & Benitez, 2004). In Fig. 2(b) we show that introduction of this plasmid in the Δcrp mutant fully restored production of CAI-1. We conclude that CRP is required to generate the CAI-1 quorum-sensing signal. We performed an electrophoresis mobility-shift experiment as described previously (Silva & Benitez, 2004) to determine if purified *V. cholerae* CRP binds the *cqsA* promoter. However, no cAMP-dependent binding could be demonstrated under the conditions used (data not shown).

Downregulation of *cqsA* was reflected in significant inhibition of *hapR* expression (Table 3). We confirmed by qRT-PCR that the Δcrp strain WL7258 expressed significantly less *hapR* mRNA (0.03 ± 0.007) compared to its wild-type precursor C7258 (2.8 ± 0.4). To determine the contribution of *cqsA* to HA/protease production, we compared isogenic *luxS::Km*, $\Delta cqsA$, Δcrp and $\Delta hapR$ mutants for protease secretion using the azocasein assay (Fig. 2c). There were no significant differences in the levels of HA/protease between the *luxS::Km* mutant M36 and wild-type (Fig. 2c). In contrast, inactivation of *cqsA* and *crp* significantly reduced HA/protease production. This result suggests that CRP regulates HA/protease production by controlling CAI-1 expression. In addition, our data suggest that, under these experimental conditions, the AI-2 signal is not essential for expression of HA/protease.

Since HA/protease is repressed by glucose (Silva & Benitez, 2004), we investigated whether CAI-1 expression is repressed by glucose, leading to diminished *hapR* mRNA. To this end, we cultured strain C7258 in HA/protease production medium. At OD_{600} 1, cultures were divided into halves; one half was used as a control and the second half transferred to a new flask containing 0.4% glucose. As expected, addition of glucose repressed production of HA/protease (data not shown). In Fig. 3 we show that glucose strongly repressed production of CAI-1, leading to lower *hapR* mRNA levels. No repression of CAI-1 production was observed after adding glucose to the spent supernatant.

Table 3. Selected genes differentially expressed in a Δcrp mutant

Abbreviations: AFC, average fold change; FDR, false discovery rate.

Gene	AFC	FDR	Description of product
VC0076	−4.1	<0.01	Universal stress protein A
VC0216	−1.8	0.01	Methyl-accepting chemotaxis protein
VC0583	−3.7	<0.01	HapR
VC0633	−1.5	0.02	OmpU
VC1034	−3.2	<0.01	Uridine phosphorylase
VC1779	−4.7	<0.01	C ₄ -dicarboxylate-binding periplasmic protein
VC1854	−3.4	<0.01	OmpT
VC2006	−1.5	0.03	Chemotaxis protein CheV
VC2125	−1.5	0.03	Flagellar motor switch protein FlhN
VC2142	−1.6	0.04	Flagellin FlaB
VC2143	−1.9	<0.01	Flagellin FlaD
VC2161	−1.5	0.03	Methyl-accepting chemotaxis protein
VC2187	−4.0	<0.01	Flagellin FlaC
VC2188	−2.5	<0.01	Flagellin core protein A
VC2190	−1.6	0.03	Flagellar hook-associated protein FlgL
VC2467	−1.7	0.02	Alternative RNAP sigma factor E
VC2614	−2.3	<0.01	cAMP receptor protein
VC2656	−2.9	<0.01	Fumarate reductase (FrdA)
VC2657	−2.6	<0.01	Fumarate reductase (FrdB)
VC2658	−2.6	<0.01	Fumarate reductase (FrdC)
VC2659	−2.8	<0.01	Fumarate reductase (FrdD)
VCA0053	−2.3	<0.01	Purine nucleoside phosphorylase
VCA0205	−2.3	<0.01	C ₄ -dicarboxylate transporter
VCA0523	−1.6	0.01	Aminotransferase (CqsA)
VCA0689	−4.3	<0.01	Hypothetical protein
VCA0690	−3.3	<0.01	Acetyl-CoA acetyltransferase
VCA0691	−3.9	<0.01	Acetoacetyl-CoA reductase
VCA0760	−2.1	<0.01	Arginine ABC transporter
VCA0867	−2.5	<0.01	OmpW
VCA0880	−1.7	0.02	Conserved hypothetical protein
VCA0881	−2.0	<0.01	Conserved hypothetical protein
VCA0882	−2.3	<0.01	Conserved hypothetical protein
VC0291	+2.2	<0.01	NifR3/Smm1 family protein
VC0827	+1.7	<0.01	Toxin co-regulated pilus biosynthesis protein H
VC2647	+2.9	<0.01	AphA

This result further confirms that CAI-1 production is subject to carbon catabolite repression. Lack of CAI-1 expression in the Δcrp mutant or its repression by exogenous glucose leads to diminished HapR levels that cannot sustain expression of HA/protease.

Consistent with the effect of CRP on CAI-1 and HapR, multiple genes regulated by quorum sensing were found to be differentially expressed in the Δcrp mutant (Table 3). For instance, hypothetical proteins VCA0880, VCA0881, VCA0882 and acetyl-CoA acetyltransferase (VCA0690) were found to be downregulated in the Δcrp mutant (Table 3) and activated by HapR (Zhu & Mekalanos, 2003).

Gene VC0291, annotated as a NifR3/Smm1 family protein, is a tRNA-dihydrouridine synthase of unknown function in

Fig. 2. Expression of *cqsA*, CAI-1 and HA/protease in Δcrp and quorum-sensing mutants. (a) *CqsA* expression. Strains C7258 (WT) and WL7258 (Δcrp) were grown in LB and cells collected at OD₆₀₀ 1.5. The *cqsA* mRNA abundance was measured by qRT-PCR. (b) CAI-1 activity. Strains C7258, WL7258 and WL7258 containing the wild-type *crp* gene *in trans* were grown as described above and the amount of CAI-1 in the cell-free supernatant was determined as described in Methods. Strain AJB61 ($\Delta cqsA$) was used as negative control. (c) HA/protease activity. Strains C7258 (wild-type), M36 (*luxS::Km*), AJB61 ($\Delta cqsA$) and WL7258 (Δcrp) were grown to stationary phase in TSB. Strain AJB51 (*hapR*) was used as a negative control. Error bars indicate standard deviations of at least three independent cultures.

V. cholerae. This protein was found to be upregulated in the Δcrp mutant (Table 3). Polar Tn5 insertions in this locus reduce the expression of the small nucleoid protein Fis (Lenz & Bassler, 2007). We did not observe differential expression of *fis* in our gene-profiling experiment. Because

Fig. 3. Repression of CAI-1 and *hapR* by glucose. Cultures of strain C7258 were grown in TSB medium and divided into halves. One half was used as a control (open bars) and the other was transferred to a new flask containing glucose (filled bars). Samples were taken after 1 and 2 h for (a) CAI-1 bioassays and (b) *hapR* expression by qRT-PCR. Error bars indicate standard deviations of at least three independent cultures. **, Significantly different from control (*t*-test, $\alpha=0.01$).

expression of *Fis* declines at high cell density (Lenz & Bassler, 2007), we used the more sensitive qRT-PCR assay to investigate the expression of this gene in the mutant. This assay confirmed that *fis* is enhanced in the Δcrp mutant (1.0 ± 0.3) compared to its wild-type precursor (0.14 ± 0.04). In a separate study we observed that the $\Delta rpoS$ mutant AJB50 grown to stationary phase expressed less *hapR* mRNA (1.9 ± 0.2) than the wild-type (3.9 ± 0.2). In *V. cholerae*, CRP activates transcription of *rpoS* in the stationary phase (Silva & Benitez, 2004). Taken together, our results show that CRP can modulate expression of HapR by multiple mechanisms which include regulation of CAI-1, *Fis* and *RpoS*.

CRP mutants make more CT and enhanced biofilm

The HapR regulator has been shown to negatively influence CT expression by repressing *AphA* and *TcpPH* (Kovachikova & Skorupski 2001, 2002). Expression of *aphA* (VC2647) and *tcpH* (VC0827) was strongly upregulated in the Δcrp mutant (Table 3). We examined whether reduced expression of *hapR* in the Δcrp mutant enhanced CT production in AKI medium. As expected, Δcrp , $\Delta hapR$ and $\Delta hapRcrp$ mutants made significantly more CT than wild-type (Fig. 4a). Furthermore, production of CT by the $\Delta hapRcrp$ double mutant was similar to that by the $\Delta hapR$ and Δcrp single mutants, suggesting that cAMP–CRP affects CT production by influencing expression of HapR.

The diminished expression of *hapR* in Δcrp mutants resulted in significantly enhanced biofilm production in LB medium compared to wild-type (Fig. 4b). The Δcrp , $\Delta hapR$ and $\Delta hapRcrp$ mutants made similar biofilms, suggesting that cAMP–CRP affects this phenotype by acting through HapR. Consistent with the biofilm results we found that the Δcrp mutant expressed elevated levels of the HapR-repressed *vpsL* exopolysaccharide biosynthesis gene (Fig. 4c). It is of note that the Δcrp mutant expressed lower *vpsL* levels than the $\Delta hapR$ mutant (Fig. 4c).

Effect of CRP on nucleoside metabolism

The finding that a mutant constitutively expressing uridine phosphorylase exhibited a superbiofilm phenotype suggested that nucleosides could play a positive role in biofilm production (Haugo & Watnick, 2002). Two genes, VC1034 and VCA0053, annotated as encoding uridine phosphorylase (Udp) and purine nucleoside phosphorylase, respectively, were downregulated in the Δcrp mutant (Table 3). We examined the expression of *udp* in wild-type, $\Delta hapR$, Δcrp and $\Delta hapRcrp$ mutants by qRT-PCR (Fig. 4d). This experiment confirmed that cAMP–CRP positively regulates *udp* expression while HapR acts as a repressor (Fig. 4d).

Effect of CRP on motility

Expression of motility has been shown to be required for intestinal colonization (Silva *et al.*, 2006). Several motility

Fig. 4. Phenotypic analysis of Δcrp mutants. (a) Cholera toxin. Strains C7258 (WT), AJB51 ($\Delta hapR$), and WL7258 (Δcrp) and WL51 ($\Delta hapRcrp$) were grown in AKI medium and the amount of CT in the culture supernatant was determined by GM₁-ELISA. (b) Biofilm formation. Strains C7258, AJB51, WL7258 and WL51 were grown in LB medium and biofilm formation was determined as described in Methods. (c) *VpsL* expression. Strains C7258, AJB51, WL7258 and WL51 were grown in LB medium and cells collected at OD_{600} 1.5. The abundance of *vpsL* mRNA was determined by qRT-PCR. Standard deviations of the mean are provided in parentheses. (d) Expression of the uridine phosphorylase (*udp*) gene. Strains C7258, AJB41, WL728 and WL51 were grown in LB as above and *udp* mRNA determined by qRT-PCR. Error bars indicate standard deviations of at least three independent cultures. *, Significantly different from wild-type (*t*-test, $\alpha=0.05$). **, Significantly different from wild-type (*t*-test, $\alpha=0.01$).

and chemotaxis genes – VC2187 (*flaC*), VC2188 (*flaA*), VC2143 (*flaD*), VC0216, VC2190 (*flgL*), VC2006 (*cheV*), VC2142 (*flaB*) and VC2125 (*flhN*) – were significantly downregulated in the Δcrp mutant (Table 3). Interestingly, *flaACD* and *flgL* have also been found to be downregulated in a $\Delta hapR$ mutant (Yildiz *et al.*, 2004). To determine the contribution of cAMP–CRP and HapR to the regulation of flagellar genes and motility we confirmed the down-regulation of *flaA* and *flaC* by qRT-PCR and compared expression of *flaA* and *flaC* in wild-type, $\Delta hapR$, Δcrp and $\Delta hapRcrp$ backgrounds. As shown in Fig. 5(a), although the $\Delta hapR$ mutant expressed lower levels of *flaA* and *flaC*, the Δcrp mutation had a more severe effect (Fig. 5a). The Δcrp mutation was epistatic to the *hapR* defect (Fig. 5a). In a swarm agar test the Δcrp mutant appeared more affected for motility compared to the *hapR* mutant, and the double mutant exhibited a cumulative effect (Fig. 5b).

cAMP–CRP affects expression of multiple genes required for intestinal colonization

In addition to flagellar and motility genes, expression of multiple genes reported to be required for intestinal colonization was significantly diminished in the Δcrp mutant. For instance, expression of genes encoding three OMPs, OmpT (VC1854) OmpW (VCA0867) and OmpU (VC0633), was significantly diminished in the mutant (Table 3). Since OmpU has been implicated in resistance to bile, organic acids and antimicrobial peptides (Mathur & Waldor, 2004; Merrell *et al.*, 2001; Provenzano & Klose, 2000; Provenzano *et al.*, 2001; Wibbenmeyer *et al.*, 2002), we confirmed that our Δcrp mutant is more sensitive than the wild-type to growth inhibition by 0.1% (w/v) sodium deoxycholate in LB medium (Fig. 6a). Expression of *rpoE* encoding the alternative sigma factor σ^E (VC2467) was also inhibited in the Δcrp mutant. We used qRT-PCR to validate that wild-type strain C7258 produced significantly more *rpoE* mRNA (0.7 ± 0.2) compared to its isogenic Δcrp mutant (0.12 ± 0.03). Growth of *V. cholerae* *rpoE* mutants is inhibited by 3% (v/v) ethanol (Kovacikova & Skorupski, 2002a). Consistent with the lower expression of *rpoE*, the Δcrp mutant was found to be more sensitive to 3% (v/v) ethanol in LB medium (Fig. 6b). Other genes potentially implicated in intestinal colonization and found to be downregulated in Δcrp mutant were VC1779 and VCA0205 encoding C₄-dicarboxylate transport proteins, the arginine ABC transporter gene VCA0760, the universal stress protein gene VC0076, and fumarate reductase subunit genes VC2656, VC2657, VC2658 and VC2659 (*frdABCD*) (Table 3). These genes have been found to be strongly induced in the rabbit ileal loop model (Xu *et al.*, 2003).

DISCUSSION

V. cholerae is known to alternate between planktonic and biofilm community lifestyles and to cycle between the human host and aquatic ecosystems. In order to adapt to

Fig. 5. Effect of cAMP–CRP and HapR on flagellar gene expression and motility. (a) Strains C7258 (WT), AJB41 ($\Delta hapR$), WL728 (Δcrp) and WL51 ($\Delta hapRcrp$) were grown in LB to OD₆₀₀ 1.5, total RNA was extracted as indicated in Methods, and *flaA* and *flaC* mRNA abundance was measured by qRT-PCR. (b) The above strains were stabbed into LB containing 0.3% (w/v) agar and incubated at 30 °C for 16 h. Error bars indicate standard deviations of at least three independent cultures.

environmental changes, *V. cholerae* needs to sense its population density (by producing autoinducer signalling molecules) and the availability of nutrients. Population density and nutrient availability are interdependent environmental signals. Consequently, response to these stimuli requires integration of multiple signal transduction pathways and global regulatory networks. Very little is known about how bacteria integrate ‘mixed’ environmental signals to evoke an adaptive response. The activity of the CRP global regulator is determined by carbon source type and availability (Kolb *et al.*, 1993). The opposing effects of

Fig. 6. Sensitivity of Δcrp mutants to bile salts and ethanol. (a) C7258 (wild-type; open bars) and WL7258 (Δcrp ; filled bars) cultures started from single colonies were grown for 16 h at 37 °C and diluted 1:100 in fresh LB containing 0.1% (w/v) sodium deoxycholate. Cultures were incubated for 6 h and growth determined by OD₆₀₀ readings. (b) Cultures were grown from single colonies as described above but diluted 1:100 in LB containing 3% (v/v) ethanol. Error bars indicate standard deviations of at least three independent cultures.

CRP on CT production (Skorupski & Taylor, 1997) and HA/protease expression (Silva & Benitez, 2004), suggest that CRP plays a key role in shifting the metabolism of *V. cholerae* from a 'virulence mode' characterized by production of CT to an 'exit mode' typified by production of the HA/protease 'detachase' activity (Finkelstein *et al.*, 1992).

Gene expression profiling of a Δcrp mutant revealed significant overlaps with the HapR-controlled regulatory network. Most importantly, production of CAI-1 synthesized by the activity of CqsA showed a nearly absolute dependence on CRP (Fig. 2a, b). The mechanism by which cAMP–CRP regulates *cqsA* is unknown. However, the absence of putative cAMP–CRP binding sites in the *cqsA* promoter suggests that cAMP–CRP could act through other regulators. Lack of CAI-1 expression in the Δcrp mutant resulted in downregulation of *hapR* and differential expression of multiple HapR-dependent genes such as *hapA*. Interestingly, inactivation of *luxS* had no effect on HA/protease expression under the conditions used. This result is in agreement with the phenotype of *luxS* mutants constructed by Zhu & Mekalanos (2003). Taken together, the results indicate that although CAI-1 and AI-2 act in parallel to activate HapR, their contribution might differ significantly under specific conditions. A possible explanation for these observations is that under conditions commonly used to grow *V. cholerae* in the laboratory the bacterium synthesizes significantly larger amounts of CAI-1 than AI-2. AI-2 is regarded as an inter-species communication molecule and it is possible that small

amounts of *V. cholerae* AI-2 could have a more pronounced effect on other bacteria than on itself. It is also possible that AI-2 could have a major effect on HapR expression under other conditions.

In other Gram-negative bacteria CRP has been reported to influence the expression of quorum-sensing-regulated genes by different mechanisms. For instance, in *Vibrio harveyi* and *V. vulnificus* CRP and LuxR concertedly activate quorum-sensing-regulated target genes (Chatterjee *et al.*, 2002; Jeong *et al.*, 2003). However, in *Vibrio fischeri* and *Pseudomonas aeruginosa*, CRP acts as an upstream regulator by directly activating transcription of LasR or LuxR (Albus *et al.*, 1997; Dunlap & Greenberg, 1988). In *E. coli*, CRP has been reported to affect quorum sensing by regulating AI-2 biosynthesis and uptake (Wang *et al.*, 2005).

Based on the finding that *cqsA* expression is significantly inhibited in Δcrp mutants, we hypothesized that CRP could affect other *hapR* phenotypes in addition to HA/protease. As shown in Fig. 4(a), Δcrp , $\Delta hapR$ and $\Delta hapRcrp$ mutants produced elevated levels of CT compared to wild-type. We note here that CRP has been reported to inhibit transcription of *tcpH* by directly binding to the *tcpPH* promoter (Kovacikova & Skorupski, 2001). However, our epistasis analysis revealed that the $\Delta hapRcrp$ mutant did not make significantly more CT than the $\Delta hapR$ mutant. This result suggests that, under our experimental conditions, direct repression of *tcpPH* by CRP does not play a major role in downregulating the TcpP/H-ToxT–CT signalling pathway.

Both Δcrp , $\Delta hapR$ and $\Delta hapRcrp$ mutants made enhanced biofilm in LB medium (Fig. 4b). Our double mutant analysis suggested that CRP and HapR could act along the same pathway to affect biofilm formation. Consistent with the biofilm data, the Δcrp mutant expressed elevated levels of the exopolysaccharide biosynthesis gene *vpsL*. VpsL has been shown to be repressed by HapR and contains a putative HapR binding site (Yildiz *et al.*, 2004). However, Δcrp and $\Delta hapRcrp$ mutants made less *vpsL* mRNA than $\Delta hapR$, suggesting that CRP could affect other factors required for maximal exopolysaccharide expression. Similar results were obtained for the exopolysaccharide biosynthesis gene *vpsA* (data not shown). We are currently investigating the interactions between cAMP–CRP, HapR and other regulators of exopolysaccharide biosynthesis.

Expression of *udp* (VC1034) has been related to biofilm formation (Haugo & Watnick, 2002). We found expression of *udp* to be strongly inhibited in the Δcrp mutant, suggesting that this gene is activated by cAMP–CRP (Fig. 4d). In addition, *udp* was found to be repressed by HapR (Fig. 4d). These results are consistent with the presence of putative HapR and cAMP–CRP binding sites in the *udp* promoter (Yildiz *et al.*, 2004; Zolotukhina *et al.*, 2003). The *udp* gene is also repressed by CytR, a repressor of biofilm formation (Haugo & Watnick, 2002). A *cytR* superbiofilm mutant expressed elevated *udp* (Haugo &

Watnick, 2002). However, our Δcrp mutant made more biofilm in spite of expressing very low levels of *udp* (Fig. 4b, d). This result suggests that derepression of *udp* in *cytR* mutants is not related to their superbiofilm phenotype.

Several flagellar genes reported to be downregulated in $\Delta hapR$ mutants (Yildiz *et al.*, 2004) were also downregulated in the Δcrp mutant, rendering this strain less motile (Fig. 5b). However, double mutant analysis suggested that CRP has its own effect on motility (Fig. 5a). FlaA is the only flagellin demonstrated to be essential for motility in *V. cholerae* (Klose & Mekalanos, 1998). There are no CRP boxes located upstream of the *flaA* ORF. Expression of *flaA* and motility is dependent on the alternative sigma factor σ^{54} (Prouty *et al.*, 2001). Studies on the *E. coli* σ^{54} -dependent *glnAp2* promoter have suggested that the cAMP–CRP complex could directly interact with the σ^{54} holoenzyme to affect gene expression without binding to a CRP box (Tian *et al.*, 2001).

It is well established that *crp* mutants, in spite of expressing elevated CT and TCP, are defective in intestinal colonization (Skorupski & Taylor, 1997). We have found that CRP positively controls multiple genes required for infant mouse colonization. These genes included flagellar genes and several OMPs that have been suggested to play a role in resistance to bile and antimicrobial peptides (Mathur & Waldor, 2004; Merrell *et al.*, 2001; Provenzano & Klose, 2000; Provenzano *et al.*, 2001; Wibbenmeyer *et al.*, 2002). OmpT and OmpW have been reported to be strongly induced in rabbit ileal loops (Xu *et al.*, 2003). Downregulation of *ompT* in the Δcrp mutant is consistent with the report of Li *et al.* (2002) showing that CRP directly activates the *ompT* promoter. As expected, the Δcrp mutants displayed increased sensitivity to sodium deoxycholate. The Δcrp mutant expressed reduced levels of *rpoE*, encoding the alternative sigma factor σ^E . Conserved functions of σ^E in Gram-negative bacteria include the synthesis, assembly and homeostasis of lipopolysaccharide and outer-membrane porins (Rhodius *et al.*, 2006). σ^E has been shown to contribute to virulence in several pathogenic bacteria, including *Salmonella*, *Haemophilus* and *V. cholerae* (Kazmierczak *et al.*, 2005; Kovacicova & Skorupski

2002a). Furthermore, expression of fumarate reductase subunits *frdABCD* was significantly diminished in the Δcrp mutant. The above genes have been found to be strongly induced in rabbit ileal loops (Xu *et al.*, 2003). It has been suggested that *frdABCD* could be required to derive energy from anaerobic respiration using alternative electron acceptors such as fumarate during growth *in vivo* (Xu *et al.*, 2003). We suggest that downregulation of these genes in the Δcrp mutant could also affect intestinal colonization. In a previous study we showed that CRP positively enhances expression of the general stress regulator RpoS (Silva & Benitez, 2004) reported to be required for intestinal colonization (Merrell *et al.*, 2000). Thus, the reduced expression of multiple factors required for intestinal colonization such as motility, OMPs, RpoE, genes required for anaerobic energy metabolism and RpoS explains the observed failure of *crp* mutants to colonize the suckling mouse intestine (Skorupski & Taylor, 1997).

In Fig. 7 we propose an integrative model showing multiple interactions by which CRP affects the *V. cholerae* life cycle. Carbon source and autoinducers act together as external signals to modulate quorum sensing. CRP serves to couple nutritional and cell density sensory information by controlling the biosynthesis of CAI-1, which contributes to activation of HapR. CRP could also influence expression of HapR through its effect on Fis and RpoS. However, these effects most likely influence expression of HapR at different stages of the *V. cholerae* growth curve. For instance, expression of *fis* is higher at low cell density (Lenz & Bassler, 2007), RpoS is expressed in the stationary phase (Silva & Benitez, 2004) and CAI-1 accumulates in the medium as the population increases. Induction of HapR by CRP negatively affects production of CT, TCP and biofilm formation. Cells within biofilm communities have been reported to be more resistant to environmental stresses and protozoan grazing (Joelsson *et al.*, 2006; Matz *et al.*, 2005). CRP can contribute to environmental stress resistance by positively influencing RpoS expression. Finally, CRP contributes to intestinal colonization by enhancing motility, RpoE, RpoS and resistance to bile. In summary, CRP plays a key role in the *V. cholerae* life cycle by integrating

Fig. 7. Model integrating the multiple regulatory interactions by which cAMP–CRP affects the *V. cholerae* life cycle. AC, adenylate cyclase; ↓, positive effect; ⊥, inhibitory effect. In this model cAMP, by binding to CRP and activating *cqsA* expression, is proposed to link carbon catabolite repression and quorum sensing.

multiple signal transduction pathways in response to environmental changes.

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