

## Transcriptional Regulation of *Vibrio cholerae* Hemagglutinin/Protease by the Cyclic AMP Receptor Protein and RpoS

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*Vibrio cholerae* secretes a Zn-dependent metalloprotease, hemagglutinin/protease (HA/protease), which is encoded by *hapA* and displays a broad range of potentially pathogenic activities. Production of HA/protease requires transcriptional activation by the quorum-sensing regulator HapR. In this study we demonstrate that transcription of *hapA* is growth phase dependent and specifically activated in the deceleration and stationary growth phases. Addition of glucose in these phases repressed *hapA* transcription by inducing *V. cholerae* to resume exponential growth, which in turn diminished the expression of a *rpoS-lacZ* transcriptional fusion. Contrary to a previous observation, we demonstrate that transcription of *hapA* requires the *rpoS*-encoded  $\sigma^S$  factor. The cyclic AMP (cAMP) receptor protein (CRP) strongly enhanced *hapA* transcription in the deceleration phase. Analysis of *rpoS* and *hapR* mRNA in isogenic CRP<sup>+</sup> and CRP<sup>-</sup> strains suggested that CRP enhances the transcription of *rpoS* and *hapR*. Analysis of strains containing *hapR-lacZ* and *hapA-lacZ* fusions confirmed that *hapA* is transcribed in response to concurrent quorum-sensing and nutrient limitation stimuli. Mutations inactivating the stringent response regulator RelA and the HapR-controlled AphA regulator did not affect HA/protease expression. Electrophoretic mobility shift experiments showed that pure cAMP-CRP and HapR alone do not bind the *hapA* promoter. This result suggests that HapR activation of *hapA* differs from its interaction with the *aphA* promoter and could involve additional factors.

*Vibrio cholerae* of serogroups O1 and O139, the causative agents of epidemic cholera, adhere to and colonize the small intestine and secrete cholera toxin (CT), which causes the major clinical symptoms of the disease. *V. cholerae* produces a soluble Zn-dependent metalloprotease (mucinase), hemagglutinin/protease (HA/protease) encoded by *hapA* (5, 13, 14, 19).

HA/protease can proteolytically activate CT A subunit (6) and the El Tor cytolysin/hemolysin (38) and can hydrolyze several physiologically important proteins such as mucin, fibronectin, and lactoferrin (14). HA/protease perturbs the paracellular barrier of cultured intestinal epithelial cells (31, 50) by acting on tight-junction-associated proteins (51) and promotes the detachment of vibrios from monolayers and mucin (4, 15). Importantly, HA/protease contributes to the reactivity of live attenuated cholera vaccine strains (3, 40).

HA/protease belongs to the thermolysin family of bacterial zinc metalloproteases (36). The HA/protease open reading frame (ORF) reveals the presence of a 24-amino-acid signal peptide followed by a 171-amino-acid N-terminal propeptide (19, 36). HA/protease, like *V. vulnificus* elastase, contains a 10-kDa C-terminal peptide (36). In *V. vulnificus* the C-terminal peptide mediates hemagglutination and is removed in an autoproteolytic reaction (37). The existence of monoclonal antibodies against HA/protease that neutralize the proteolytic but not the hemagglutinating activity suggests a domain structure similar to that of *V. vulnificus* elastase (21). HA/protease is secreted via the *V. cholerae* type II secretion pathway at the cell pole of the single polar flagellum (42, 43).

Expression of *hapA* requires transcriptional activation by HapR (24), a homologue of *V. harveyi* LuxR. The regulators LuxO and HapR coordinate cell density-dependent expression of CT, toxin-coregulated pilus (TCP), HA/protease production, motility, and biofilm formation (54). At low cell density, the active form of LuxO (phospho-LuxO) represses *hapR*, a condition conducive to the expression of *aphA* (26, 54). At high cell density, LuxO is inactive and HapR is expressed to activate *hapA* and repress *aphA*, encoding an activator of *tcpPH* (26, 47, 54). It appears that *V. cholerae* harbors three quorum-sensing systems (34). System 1 consists of a CqsA-dependent autoinducer, CAI-1, and its sensor, CqsS (34). System 2 consists of the LuxS-dependent autoinducer CAI-2 and its sensor, LuxPQ, similar to *V. harveyi* (34). The existence of a system 3 has been inferred genetically (34). The three systems appear to feed the quorum-sensing signal to LuxO/HapR in order to regulate virulence gene expression (34).

Production of HA/protease is influenced by environmental factors other than cell density (2, 45). Secretion of HA/protease is strongly enhanced by nutrient limitation (2). A *crp* mutant secreted less HA/protease (2). It was also reported that a *rpoS* insertion mutant secreted less HA/protease (53). However, the protease defect was not fully complemented in *trans*, and it was not established whether the mutation affected the production or secretion of HA/protease (53). In a later study using a hemagglutination assay for HA/protease, we did not observe the above *rpoS* dependency (2). These findings suggest that the expression of *hapA* is more complex and requires the integration of multiple environmental signals through more than one global regulator.

Starvation stress regulators such as *relA* and the *rpoS*-encoded stationary-phase  $\sigma^S$  factor affect intestinal colonization and virulence in *V. cholerae* (18, 32, 46). In *Escherichia coli*, the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics
<i>V. cholerae</i> strains	
C7258	Wild type, E1 Tor (Peru isolate, 1991)
C6709-1	Wild type, E1 Tor (Peru isolate, 1991)
C6706	Wild type, E1 Tor (Peru isolate, 1991)
N16961	E1 Tor, <i>hapR</i> (naturally occurring frameshift in <i>hapR</i> )
AC-V66	C6709-1, <i>lacZ::res-tet-res</i> (A. Camilli, Tufts University) (29)
KSK394	C6706, <i>crp::Km<sup>r</sup></i> (K. Skorupski, Dartmouth Medical School) (46)
DSM-V491	C6709-1, $\Delta$ <i>rpoS</i> (A. Camilli, Tufts University) (32)
AJB2	AC-V66, <i>hapA::hapA-lacZ</i> , Amp <sup>r</sup> (this study)
AJB3	KSK394, <i>hapA::hapA-lacZ</i> , Amp <sup>r</sup> (this study)
AJB19	C7258 <i>relA::pCVD<math>\Delta</math>relAK</i> (this study)
AJB216	N16961, $\Delta$ <i>aphA</i> (this study)
AJB231	AC-V66, $\Delta$ <i>aphA</i> (this study)
AJB25	DSM-V491, <i>hapR::hapR-lacZ</i> (this study)
AJB26	AC-V66, <i>hapR::hapR-lacZ</i> (this study)
AJB29	AC-V66, <i>rpoS::rpoS-lacZ</i> (this study)
<i>E. coli</i> strains	
DH5 $\alpha$	F <sup>-</sup> $\phi$ 80 <i>lacZ</i> M15 $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>deoR recA1 endA1 hsdR17</i> ( <i>r<sub>K</sub><sup>-</sup> m<sub>R</sub><sup>+</sup></i> ) <i>phoA supE44 <math>\lambda</math><sup>-</sup> thi-1 gyrA96 relA1</i>
SY327 $\lambda$ Pir	$\Delta$ ( <i>lac pro</i> ) <i>argE</i> (Am) <i>rif nalA recA56</i> ( $\lambda$ <i>pirR6K</i> ) (35)
SM10 $\lambda$ Pir	<i>thi thr leu tonA lacY supE recA::RP4-2Tc::Mu</i> ( $\lambda$ <i>pirR6K</i> ) (35)
Plasmids	
pBADHisB	Expression vector with araBAD promoter, Amp <sup>r</sup> (Invitrogen)
pALTERex <sup>2</sup>	<i>In vitro</i> mutagenesis vector, T7 promoter, Tet <sup>r</sup> (Promega Corp.)
pGP704	Suicide vector ( <i>oriR6K, mobRP4, Amp<sup>r</sup></i> ) (35)
pCVD442	Suicide vector ( <i>oriR6K, mobRP4, sacB, Amp<sup>r</sup></i> ) (11)
pHapLac11	Plasmid vector containing transcriptional <i>hapA-lacZ</i> fusions (45)
pHapRLac2	Plasmid vector containing transcriptional <i>hapR-lacZ</i> fusions (45)
pGPHap10	KpnI-PstI <i>hapA-lacZ</i> fusion from pHapLac11 in pGP704 (this study)
pGPHap13	KpnI-PstI <i>hapR-lacZ</i> fusion from pHapRLac2 in pGP704 (this study)
pCH2	pACYC184 containing 3.2-kb HindIII <i>hapA</i> insert (19)
pBADHapR9	HapR ORF cloned in pBADHisB (this study)
pBADCRP7	CRP ORF cloned in pBADHisB (this study)
pCVD $\Delta$ relA	1,077-bp internal <i>relA</i> fragment cloned in pCVD442 (this study)
pCVD $\Delta$ relAK	pCVD $\Delta$ relA with Km <sup>r</sup> in unique NsiI site of <i>relA</i> (this study)
p $\Delta$ AphA	5' SphI-BamHI and 3' BamHI-SacI <i>aphA</i> fragments in pUC19 (this study)
pCVD $\Delta$ AphA	SphI-SacI $\Delta$ AphA fragment from p $\Delta$ AphA cloned in pCVD442 (this study)
pRpoS5	SphI-HindIII <i>rpoS</i> promoter fragment cloned in pUC19 (this study)
pRpoSlac5	SphI-HindIII <i>rpoS</i> DNA replacing <i>hapR</i> promoter in pHapRLac2 (this study)
pGPRpoSlacZ	XbaI-ScaI fragment from pRpoSlac5 cloned in pGP704 (this study)
pJB43	428-bp <i>hapA</i> 5' fragment in pALTERex <sup>2</sup> (this study)

intracellular level of  $\sigma^S$  is controlled at the level of transcription, translation, and protein stability (20, 48). The *relA* gene is the genetic determinant of the stringent response. The *relA*-catalyzed increase in the amount of guanosine tetraphosphate (ppGpp) leads to rapid inhibition of stable RNA synthesis and ultimately to growth arrest (8). A *V. cholerae relA* mutant showed lower levels of CT and TCP, altered levels of OmpU and OmpT porins, reduced motility, and a 1,000-fold reduction in infant-mouse colonization (18). The effect of *relA* inactivation on *hapA* expression was not examined. Quorum-sensing regulators and *rpoS* cross-regulate their expression in *P. aeruginosa* (49), but the extent to which such interactions occur in *V. cholerae* is unknown.

In the present study we show that transcription of *hapA* is growth phase dependent, requires the *rpoS*-encoded  $\sigma^S$  factor, and is enhanced by cyclic AMP (cAMP) receptor protein (CRP). CRP enhanced the transcription of *hapR* and *rpoS*, which in turn are required for HA/protease expression. Glucose repressed the *hapA* gene by inducing cells to resume

exponential growth and lowering the level of *rpoS* transcription. Finally, we used strains containing chromosomal *hapA-lacZ* and *hapR-lacZ* transcriptional fusions to demonstrate that *hapA* is transcribed in response to concurrent cell density and nutrient limitation stimuli.

## MATERIALS AND METHODS

**Strains and media.** The *V. cholerae* and *E. coli* strains, plasmids, and primers used in this work are listed and described briefly in Tables 1 and 2. Strains were grown in Bacto tryptic soy broth (TSB) (Becton, Dickinson & Co.) or Luria-Bertani (LB) broth at 37°C with agitation (250 rpm). When indicated, liquid medium was supplemented with 0.4% D-glucose. The sensitivity to 3-amino-1,2,4-triazole (AT) was determined by using M9 minimal medium supplemented with glucose (0.2%), all amino acids except histidine (4  $\mu$ g/ml), adenine (1 mM), thiamine (1 mM), and AT (15 mM). Sensitivity to inhibition by serine, methionine, glycine, and leucine (SMGL) was tested in experiments with M9 medium supplemented with glucose (0.2%), SMGL (100  $\mu$ g/ml each), adenine (50  $\mu$ g/ml), thymine (50  $\mu$ g/ml), and calcium pantothenate (1  $\mu$ g/ml). Plasmid DNA was introduced into *V. cholerae* by electroporation (30). Culture media were supplemented with ampicillin (100  $\mu$ g/ml), tetracycline (1  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) (20  $\mu$ g/ml), or

TABLE 2. Primers used in this study

Primer name	Sequence
AphA40	5'-CGGGAATGCGCAATACTGGTTAACA
AphA207	5'-CGCTTATCACAGAGGGAACGTAGT
AphA1409	5'-GCTGGATCCGAAGTGGTTGAAGAAT
AphA2441	5'-GAAGAGCTCGCCAGTTTTACCATG
AphA248	5-CCATTAATGAAGGACACCAAGCGC
AphA1169	5'-GAAGGATCCGATATACCTGCTGATG
AphA1742	5'-GAGCAGTTTAAATGGTTCGTTCCGAGA
AphA940	5'-GGGTGACATAAGCAGCCGAATTTTG
Cap123	5'-GCAGCCATGACAAAAACGCGTAAC
Cap249	5'-GCCGATATGGAGAAACAGTAGAGA
Crp892	5'-GTTTACCCTGGCCAGAGAT
Crp794	5'-CTTGGCAGTGTACTTTGATTGCA
Crp367	5'-CATAAGTACCCATCAAAAAGCACGC
Crp304	5'-GGCGGATCCATGGTTCTAGGTAAC
Crp945	5'-GGCAAGCTTACACGAGACGGGTTAT
HapA459	5'-ACATGCATGCCTGCTTTCTATCGA
HapA502	5'-ACATGCATGCACACGTAGAGTTTACA
HapA664	5'-GGCCAAGCTTCAATTTCTCAATCCTAG
HapA456	5'-GGCGGATCCCTTTCTGGCTTTCTTAT
HapA875	5'-GCGAATTCCTTGTACCTTGCT
HapR1100	5'-GCCAACTTATACACTGCTTCT
HapR1046	5'-ATGGAGTAGAAGATGCCGTGGAAC
HapR589	5'-CGCCTCAAAAACGCAAACTACAAC
HapR552	5'-AAACTGCAGGACGCATCAACTGAAAA
HapR1158	5'-CCGGAATTCGCTGCCCAAGAACTA
RelA842	5'-GTTCTAGAGGTGATTAAGCTTGCCG
RelA1895	5'-GTTGCATGCCCGCTTCGAGATT
RpoS1226	5'-CAGCAGTGCCTTATCTCCAT
RpoS1003	5'-TGTTTGGTTCATCAGCGCACGTT
RpoS576	5'-CGATTTTGAAGATGAAGCACTGGAAG
RpoS228	5'-AAAGCATGCATGTTGAACCTGTCGG
RpoS549	5'-GGGAAGCTTTTGGTTACGGTATTG
RecA1061	5'-CGCTTTACCTTGGCCGATTT
RecA578	5'-GTGCTGTGGATGTCATCGTTGTTG
RecA863	5'-CCACCACTTCTTCGCTTCTTTGA

polymyxin B (100 U/ml) as required. Strains AC-V66 (29), KSK394 (46), and DMS-V491 (32) do not produce detectable endogenous  $\beta$ -galactosidase activity and were used as hosts for *rpoS*, *hapR*, or *hapA-lacZ* transcriptional fusions. Growth was monitored by reading the optical density at 600 nm (OD<sub>600</sub>).

**Cloning of *V. cholerae* genes and regulatory sequences.** Chromosomal DNA was purified from *V. cholerae* as described previously (1). Unless otherwise specified, *V. cholerae* genes and regulatory sequences were amplified from genomic DNA of strain C6709-1 by using the Advantage PCR system (Clontech). All primers were designed based on the DNA sequence of the *V. cholerae* N16961 genome downloaded from The Institute for Genomic Research database. The ORFs encoding HapR and CRP were amplified with primers HapR552 plus HapR1158 and Crp304 plus Crp945, respectively. The *V. cholerae* *rpoS* promoter region was amplified with primers RpoS228 and RpoS549. An internal *relA* fragment was amplified with primers RelA842 and RelA1895. A 201-bp fully active *hapA* promoter fragment was amplified with primers HapA459 and HapA664. A second, inactive 158-bp *hapA* promoter fragment lacking a putative CRP binding site was amplified with primers HapA502 and HapA664. An *aphA* promoter fragment containing a HapR binding site was amplified with primers AphA40 and AphA207. An *E. coli* arabinose (*araBAD*) promoter fragment containing a cAMP-CRP binding site was amplified from pBADHisB with primers Cap123 and Cap249. All amplification products were directionally cloned in pUC19, and both DNA strands were sequenced using the M13 forward and reverse sequencing primers at the Emory University School of Medicine DNA Core facility.

**Construction of *V. cholerae* strains containing chromosomal *hapA-lacZ*, *hapR-lacZ*, and *rpoS-lacZ* transcriptional fusions.** Construction of *hapA-lacZ* and *hapR-lacZ* transcriptional fusions was described previously (45). KpnI-PstI fragments containing the *hapA-lacZ* fusion from pHaplac1 and the *hapR-lacZ* fusion from pHapRlac2, respectively (Table 1), were subcloned in a suicide vector, pGP704 (35), digested with the above enzymes to generate suicide plasmids pGPHap10 and pGPHap13 (Table 1). To construct *V. cholerae* strains containing chromosomal *rpoS-lacZ* fusions, the SphI-HindIII insert in pRpoS5

(Table 1) was subcloned in pHapRlac2, replacing *hapR* promoter DNA to generate pRpoSlacZ. Next, the *rpoS-lacZ* fusion was extracted as an XbaI-ScaI fragment and ligated to pGP704 digested with the above enzymes to create the suicide vector pGPRpoSlacZ (Table 1). All suicide vector were constructed in SY327 $\lambda$ Pir, electroporated to SM10 $\lambda$ Pir, and transferred by conjugation to receptor *V. cholerae* strains, and exconjugants were selected in LB agar containing ampicillin and polymyxin B. Suicide vector pGPHap10 was transferred to AC-V66 and KSK293 to generate strains ABJ2 and ABJ3, respectively. Suicide vector pGPHap13 was transferred to AC-V66 and DSM-V491 to create strains ABJ26 and ABJ25, respectively. Finally, pGPRpoSlacZ was transferred to strains AC-V66 to generate ABJ29 (Table 1). Integration into the chromosomal *hapA* or *hapR* locus was verified by Southern hybridization with a digoxigenin (DIG)-labeled *hapA* HindIII fragment isolated from plasmid pCH2 (19) or the *hapR* ORF isolated from pBADHapR9, respectively. Correct integration was confirmed by detection of *hapA* or *hapR* in a BglIII fragment with a molecular weight higher than in the precursor strains. Correct integration in the *rpoS* locus in strain ABJ29 was verified by Southern hybridization with a DIG-labeled *rpoS* fragment from pRpoS5. Integration in the *rpoS* locus of ABJ29 was confirmed by the detection of anticipated HindIII junction fragments.

**Construction of  $\Delta$ *aphA* mutants.** The 5' and 3' fragments of the *aphA* gene were amplified from strain C6709-1 using primer combinations AphA248 plus AphA1169 and AphA1409 plus AphA2441, respectively. The amplified DNAs were sequentially cloned as SphI-BamHI and BamHI-SacI fragments in pUC19, creating an *aphA* gene with an internal deletion and a frameshift ( $\Delta$ *aphA*) (Table 1). The deleted and frameshifted *aphA* gene was transferred to pCVD442 (11) as a SphI-SacI fragment to generate pCVD $\Delta$ *aphA*. This plasmid was transferred to *E. coli* SM10 $\lambda$ pir and mobilized by conjugation to *V. cholerae* N16961 and AC-V66. Exconjugants were selected in LB medium containing ampicillin and polymyxin. Integration into the *aphA* locus was verified by Southern hybridization using the DIG-labeled 3' *aphA* amplification product cloned in p $\Delta$ *aphA*. Correct integration was confirmed by the detection of two HindIII junction fragments in exconjugants instead of the single HindIII fragment detected in precursor strains. Exconjugants from N16961 and AC-V66 were allowed to segregate in LB medium and plated in LB agar containing 5% sucrose. Sucrose-resistant clones were tested for ampicillin sensitivity and screened for the deleted *aphA* allele by PCR using primers AphA940 plus AphA1742. Segregants ABJ216 and ABJ231 were found to contain the deleted *aphA* gene.

**Disruption of the activity domain of *V. cholerae* *relA*.** A 1,077-bp *V. cholerae* *relA* fragment was amplified as described above and cloned in suicide vector pCVD442 (11) as a SphI-XbaI fragment to generate pCVD $\Delta$ *relA*. A PstI fragment containing the Km<sup>r</sup> gene from plasmid pUC4K (Pharmacia) was subsequently inserted in a unique NsiI site located within the highly conserved enzyme activity domain (between essential residues G251 and H354) to create pCVD $\Delta$ *relAK*. This plasmid was transferred by conjugation to *V. cholerae* C7258 as described above to generate exconjugant ABJ19. The organization of the *relA* locus in exconjugant ABJ19 was determined by Southern hybridization analysis of NsiI digests using the DIG-labeled amplified 1,077-bp *relA* fragment.

**RNase protection assays.** Total RNA was isolated using the RNeasy kit (Qiagen Laboratories). A 5' fragment of *hapA* was amplified using primers HapA456 plus HapA875 and cloned in pALTERex<sup>2</sup> (Promega Corp.) to generate plasmid pJB43. A 428-bp radiolabeled riboprobe was synthesized by *in vitro* transcription with T7 RNA polymerase in the presence of 50  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP (800 Ci/mmol; Amersham). The riboprobe was purified by polyacrylamide gel electrophoresis, and 2  $\times$  10<sup>4</sup> to 8  $\times$  10<sup>4</sup> cpm was annealed with 10  $\mu$ g of total RNA. Single-stranded RNA was degraded with RNase A and RNase T<sub>1</sub> by using the Ambion RPA III kit, and protected fragments were separated in an 8 M urea-5% polyacrylamide gel. After autoradiography, a single 318-bp protected fragment was detected in *V. cholerae* strains expressing *hapA* (Fig. 1).

**RT-PCR assays.** Reverse transcription PCR (RT-PCR) analysis was performed using the Titanium one-step RT-PCR kit (Clontech). Briefly, 20 ng of total RNA was used as the template for cDNA synthesis. The following primer combinations were used: RpoS1223, RpoS1003, and RpoS576 for *rpoS* mRNA; HapR1100, HapR1046, and HapR589 for *hapR* mRNA; Crp892, Crp794, and Crp367 for *crp* mRNA; and RecA1061, RecA578, and RecA863 for *recA* mRNA. A control without reverse transcriptase was used for each reaction to exclude chromosomal DNA contamination. For quantitative comparisons, RNA samples were analyzed by real time RT-PCR using the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad Laboratories). Relative expression values (*R*) were calculated using the equation  $R = 2^{-(\Delta C_t \text{ target} - \Delta C_t \text{ reference})}$ , where *C<sub>t</sub>* is the fractional threshold cycle. The *recA* mRNA was used as reference.

**Expression and purification of His-HapR and His-CRP.** The ORFs encoding HapR and CRP were amplified from C6709-1 as described above and subcloned in pBADHisB (Invitrogen) to generate pBADHapR9 and pBADCRP7, respec-

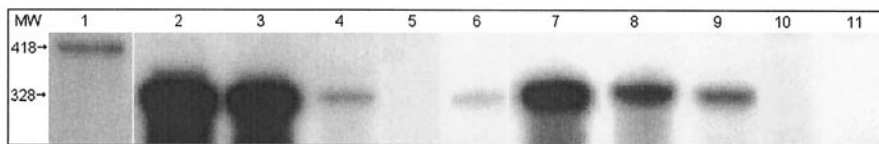


FIG. 1. RNase protection assay analysis of *hapA* expression. *V. cholerae* strains were grown in TSB medium to an OD<sub>600</sub> of 2 (deceleration phase). At this point, 10-ml aliquots were withdrawn for total-RNA extraction (lanes 2 to 6) and the remaining culture was further incubated for 16 h (late stationary phase) (lane 7 to 11). Lanes: 1, intact probe (RNase-minus control); 2 and 7, C6706; 3 and 8 C6709-1; 4 and 9, KSK394 (*crp*); 5 and 10; N16961 (*hapR*); 6 and 11; DSM-V491 (*rpoS*). MW, molecular size in base pairs.

tively. Both genes were expressed from the arabinose (*araBAD*) promoter as His<sub>6</sub> fusions. His<sub>6</sub>-HapR and His<sub>6</sub>-CRP were purified to homogeneity in ProBond columns as specified by the manufacturer (Invitrogen), for induction, cell disruption (sonication), and affinity purification. Purity was confirmed by the appearance of a single band in a Coomassie blue-stained SDS-PAGE gel that reacted with anti-Xpress antibodies (Invitrogen).

**DNA binding assays.** DNA binding assays were conducted using the DIG gel shift procedure (Roche). A 201-bp fully active and HapR-dependent *hapA* promoter fragment and a 158-bp 5' deletion fragment lacking promoter activity were amplified as described above. A 162-bp *aphA* fragment containing a HapR binding site (26) was used as a positive control. Similarly, a 126-bp fragment containing a cAMP-CRP binding site from pBADHisB was used as a positive control for cAMP-CRP binding. Fragments were labeled with DIG and terminal transferase, and binding reactions were conducted as recommended by the kit manufacturer, using 10 ng of labeled DNA. DNAs were separated in a 5% nondenaturing polyacrylamide gel and processed for chemiluminescence detection.

**Western blot analysis.** The presence of HA/protease in *V. cholerae* supernatants was detected with an anti-HA/protease serum and peroxidase-conjugated anti-rabbit immunoglobulin G as described previously (2).

**Enzyme assays.** β-Galactosidase activities were measured as described by Miller (33), using the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Specific activities are given in Miller units (1,000 OD<sub>420</sub> t<sup>-1</sup> v<sup>-1</sup> OD<sub>600</sub><sup>-1</sup>), where *t* is reaction time and *v* is the volume of enzyme extract per reaction.

RESULTS

**Transcription of *hapA* requires *rpoS* and is enhanced by CRP.** As shown in Fig. 1 (lanes 4 to 6), very little or no *hapA* mRNA could be detected in *crp*, *hapR*, and *rpoS* mutants in the growth deceleration phase. As cells progressed into the late stationary phase, expression of *hapA* became less dependent on CRP (Fig. 1, compare lanes 7 and 9) but remained strongly dependent on *hapR* and *rpoS* (lane 10 and 11). Interestingly, lower levels of *hapA* mRNA were detected in cells grown to late stationary phase (Fig. 1, compare lanes 7 and 8 to lanes 2 and 3). We considered the possibility that overexpression of σ<sup>S</sup> in the late stationary phase could diminish the expression of *hapR*. Strains AJB25 and AJB26 contain a *hapR-lacZ* fusion integrated in a *rpoS* and *rpoS*<sup>+</sup> genetic background, respectively (Table 1). When these strains were grown to late stationary phase, the Δ*rpoS* mutant expressed slightly higher levels of the *hapR-lacZ* fusion (21.7 ± 2.6 and 13.7 ± 2.1 Miller units for AJB25 and AJB26, respectively).

To study the role of CRP and *rpoS* in *hapA* transcription, we first considered the possibility that HapR, CRP, and RpoS could cross-regulate their transcription. We determined the production of *rpoS*, *hapR*, and *crp* mRNA in wild-type strains and isogenic mutants (Fig. 2). Lower levels of *rpoS* and *hapR* mRNA were detected in *crp* mutant KSK394 than in its precursor, C6706 (Fig. 2A). No differences were detected under these conditions (growth deceleration phase) for *crp* and *hapR* mRNA production in a Δ*rpoS* mutant compared to its isogenic

precursor (Fig. 2B). Because conventional RT-PCR tends to be biased against samples with more abundant transcripts, total RNA from C6706 and KSK394 was analyzed by real-time RT-PCR for *rpoS*, *hapR*, and *recA* mRNA. As shown in Fig. 2C, strain KSK394 produced substantially lower levels of both *rpoS* and *hapR* mRNA.

**Glucose represses *hapA* by inducing *V. cholerae* to resume exponential growth.** Exconjugant AJB3 (*crp* mutant) produced five times less β-galactosidase activity than did AJB2 (*crp*<sup>+</sup>), confirming that the expression of *hapA* is enhanced by CRP. However, addition of glucose at the growth deceleration phase

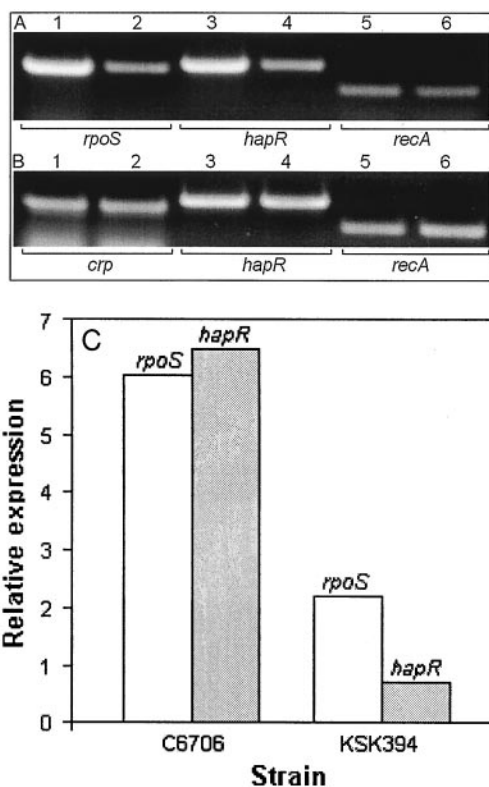


FIG. 2. Transcription of *rpoS*, *hapR*, and *crp* in wild-type and isogenic regulatory mutants. *V. cholerae* strains were grown in TSB to an OD<sub>600</sub> of 2, and RT-PCR was conducted as described in Materials and Methods. (A) Lanes: 1, 3, and 5, C6706 (*crp*<sup>+</sup>); 2, 4, and 6, KSK394 (*crp*). (B) Lanes: 1, 3, and 5 C6709-1 (*rpoS*<sup>+</sup>); 2, 4, and 6, DMS-V491 (*rpoS*). The mRNA detected in each lane is written below each panel. (C) Real-time RT-PCR. RNA extracted from three cultures of C6706 and KSK394 was analyzed for the relative expression of *rpoS* and *hapR* (targets) with *recA* mRNA as reference.

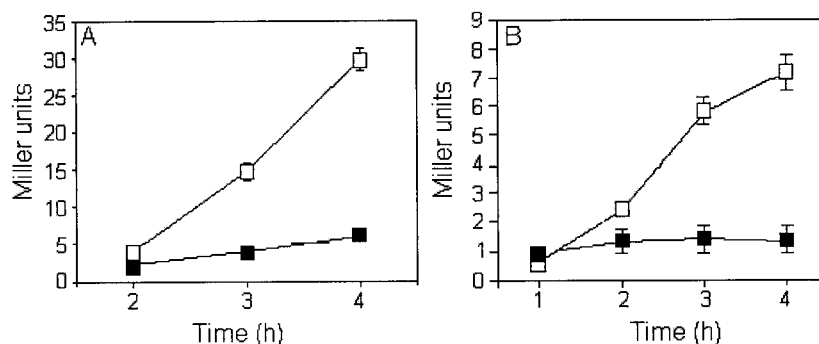


FIG. 3. Glucose repression does not require CRP. Strains AJB2 (A) and AJB3 (B) containing a *hapA-lacZ* transcriptional fusion inserted in the *hapA* locus in a *crp*<sup>+</sup> (AC-V66) and *crp* (KSK394) background, respectively, were grown in TSB medium to an OD<sub>600</sub> of 1. Cultures were divided in half and further incubated for 4-h with (■) and without (□) glucose. Samples were taken hourly for enzyme assays and OD<sub>600</sub> determinations. Each value represents the average of three independent cultures. Error bars indicate the standard deviation of the mean.

repressed the *hapA* promoter in both strains, demonstrating that glucose repression of *hapA* is not mediated by CRP (Fig. 3). Repression of *hapA* by glucose in TSB medium was paralleled by stimulation of growth (Fig. 4A and B). In Fig. 4C we show that addition of glucose reduced the expression of a *rpoS-lacZ* transcriptional fusion in *V. cholerae*.

We next considered whether cells induced to enter the stationary phase at lower cell densities could transcribe *hapA*. To test this possibility, we performed a nutritional downshift experiment. A low level of *hapR* mRNA was detected by RT-PCR in exponentially growing *V. cholerae* AJB2 cells at OD<sub>600</sub> values as low as of 0.2 (data not shown). *V. cholerae* AJB2 was grown in rich TSB medium to an OD<sub>600</sub> of 0.5, at which *hapA* expression is not detected, and half of the culture was collected and resuspended in 0.25× TSB. As shown in Fig. 5A, the nutritional downshift had a twofold effect. First, expression of *hapA* was diminished due to lower levels of HapR at the shift point, indicating that nutrient limitation alone does not fully activate *hapA*. Second, *hapA* expression could be detected at a lower optical density. To determine if the expression of *hapA* at a lower optical density was due to activation of *hapR* or *hapA*, strains AJB2 (*hapA-lacZ*) and AJB26 (*hapR-lacZ*) were grown to late stationary phase in TSB medium of different

strengths. While *hapA* promoter activity was enhanced by nutrient limitation, the activity of the *hapR* promoter remained constant (Fig. 5B). We conclude that that expression of *hapA* requires two concurrent environmental stimuli: high cell density and entry to stationary phase due to nutrient limitation.

**Expression of *hapA* does not require RelA or AphA.** In *E. coli*, ppGpp enhances the transcription of *rpoS* (8, 17, 41) and many *rpoS*-dependent genes (28). Therefore, we examined the role of *relA* in the expression of *hapA*. Southern hybridization showed that strain AJB19 contains two inactive *relA* alleles: one containing a Km<sup>r</sup> insertion in the N-terminal activity domain and a second allele lacking the entire N terminus (data not shown). As expected from the phenotype of *E. coli relA* mutants (41), strain AJB19 was sensitive to AT and amino acids SMGL in combination (data not shown). Nevertheless, AJB19 produced wild-type levels of HA/protease (Fig. 6, lane 2). We also considered the possibility that HapR could control the transcription of *hapA* through the regulator AphA, the only known target for HapR (26, 27). Since transcription of *aphA* is repressed by HapR at high cell density (26), we speculated that *aphA* could encode a repressor of *hapA*. This hypothesis predicts that inactivation of *aphA* should restore HA/protease production in *hapR* mutant strain N16961 and

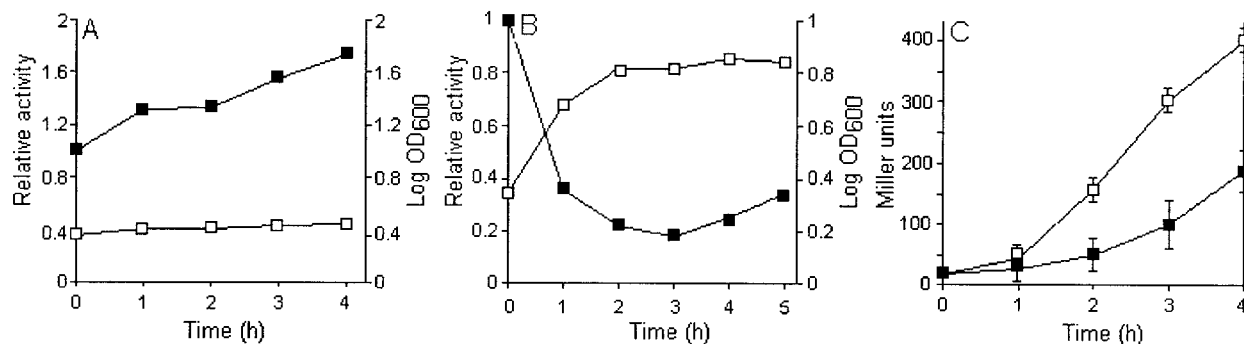


FIG. 4. Correlation between glucose repression and growth stimulation. (A and B) Strain AJB2 was grown to an OD<sub>600</sub> of 2, and the culture was divided in half. Glucose was added to one half (B), and the other half was used as a control (A). Samples were taken at 1-h intervals for  $\beta$ -Galactosidase activity (■) and OD<sub>600</sub> readings (□). (C) Strain AJB29 containing a *rpoS-lacZ* transcriptional fusion was grown in TSB to an OD<sub>600</sub> of 1, and the culture was divided in half. Half was supplemented with glucose (■), and half was used as a control (□). Relative activities in panels A and B refer to the Miller units at the time of glucose supplementation. Final Miller units reported are the mean of three independent cultures. Error bars indicate the standard deviation of the mean.

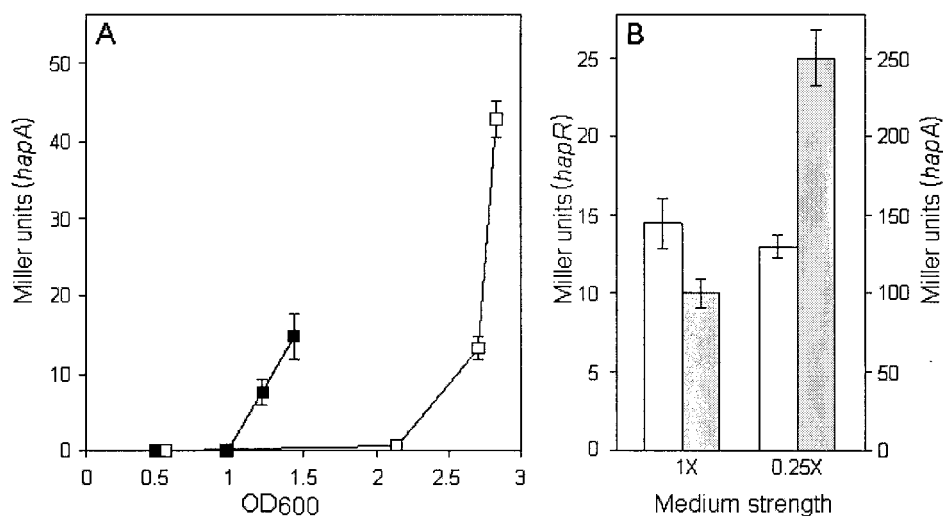


FIG. 5. Effect of a nutritional downshift on *hapA* promoter activity. (A) *V. cholerae* AJB2 was grown to an OD<sub>600</sub> of 0.5. Half of the culture was centrifuged, and the cells were resuspended in 0.25 × TSB (■); the remaining cells were kept in 1 × TSB (□). Cultures were analyzed for β-galactosidase activity at different time points. (B) Overnight cultures of strain AJB26 (*hapR-lacZ*) (open bars) and AJB2 (*hapA-lacZ*) (shaded bars) were diluted in TSB medium of different strengths and incubated at 37°C until late stationary phase. Cultures were analyzed for *hapR*- and *hapA*-driven β-galactosidase expression. The final Miller units reported represent the mean of three independent cultures. Error bars indicate the standard deviation of the mean.

enhance protease production in *hapR*<sup>+</sup> strain AC-V66. We constructed two strains, each containing a deletion and frame-shift mutation in the *aphA* gene. Strain AJB216, derived from N16961, and strain AJB231, derived from AC-V66, produced significantly reduced levels of CT as a result of the *aphA* mutation (26) (data not shown). However, the *aphA* mutation in AJB216 did not restore HA/protease production in N16961 (Fig. 6, lane 4) and AJB231 did not express significantly higher levels of HA/protease than AC-V66 (lane 6).

**HapR and CRP alone do not bind *hapA*.** We conducted electrophoretic mobility shift assays using the 201-bp minimal but fully active and HapR-dependent *hapA* promoter fragment and the a 158-bp inactive fragment which lacks a DNA sequence with dyad symmetry, resembling a cAMP-CRP binding site. Binding experiments revealed that purified His<sub>6</sub>-HapR and His<sub>6</sub>-CRP do not bind the active *hapA* promoter. Both proteins were capable of shifting the positive control fragment containing a HapR binding site from *aphA* and a CRP site from the arabinose operon (Fig. 7). No electrophoretic mobility shift was detected by adding His<sub>6</sub>-CRP and His<sub>6</sub>-HapR together to the binding-reaction mixture.

## DISCUSSION

Very little is known about how environmental factors other than cell density control the transcription of *hapA* and about the role of CRP and RpoS in this process. Barely detectable *hapA* mRNA is synthesized in the growth deceleration phase in *crp*, *hapR*, and *rpoS* mutants (Fig. 1). Strong *rpoS* dependency was also confirmed by the finding that strain DSM-V491 does not support the expression of *hapA-lacZ* fusions forming white colonies in X-Gal medium. In a previous study, we observed that Δ*rpoS* mutant DSM-V491 secreted three times less azo-casein activity but normal levels of soluble hemagglutinating activity. Since the HA/protease carries both activities, we concluded that HA/protease was not affected in this mutant. In the above study, strains were incubated at 30°C for longer periods (> 24 h). It is possible that *rpoS* mutants could release other proteases and materials with hemagglutinating activity on prolonged incubation in the stationary phase due to their stress survival defect (53). In Fig. 6 (lane 7) we show that no HA/protease-cross-reacting material could be detected in concentrated supernatants of strain DSM-V491.

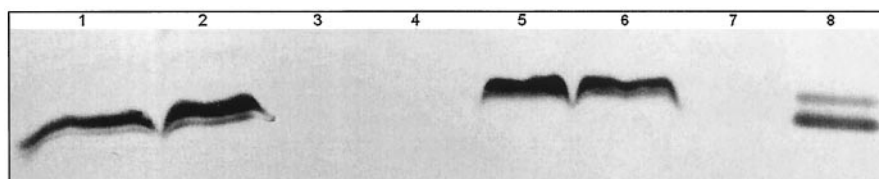


FIG. 6. Western blot analysis of HA/protease expression in *V. cholerae* mutants. Supernatants from *V. cholerae* strains grown to saturation in TSB medium were concentrated in Centricon-10 centrifugal filters (Amicon Bioseparations), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) and transferred to a polyvinylidene difluoride membrane. Lanes: 1, C7258; 2, AJB19; 3, N16961; 4, AJB216; 5, AC-V66; 6, AJB231; 7, DSM-V491; 8, pure HA/protease.

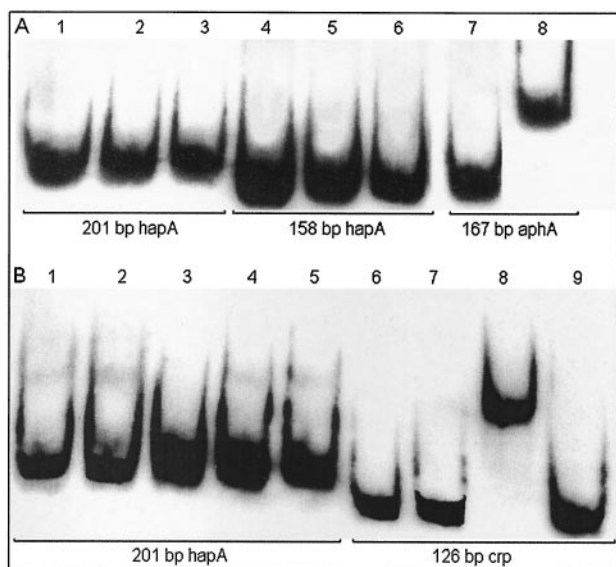


FIG. 7. DNA binding assays. (A) His<sub>6</sub>-HapR. DIG-labeled DNA fragments (10 ng) were incubated with no protein (lane 1), 64 ng of protein (lane 2), 128 ng of protein (lane 3), no protein (lane 4), 64 ng of protein (lane 5), 128 ng of protein (lane 6), no protein (lane 7), and 64 ng of protein (lane 8) (B) His<sub>6</sub>-CRP. DIG-labeled DNA fragments (10 ng) were incubated with no protein (lane 1), 50 ng of protein and cAMP (lane 2), 50 ng of protein (lane 3), 500 ng of protein and cAMP (lane 4), 500 ng of protein (lane 5), no protein (lane 6), no protein with cAMP (lane 7), 500 ng of protein and cAMP (lane 8), and 500 ng of protein (lane 9). The concentration of cAMP was 500  $\mu$ M.

Transcription of *hapA* is strongly enhanced by CRP (Fig. 1). As cells progressed into the late stationary phase, expression of *hapA* became less dependent on CRP (Fig. 1, lane 9) but remained strongly dependent on *hapR* and *rpoS* (lanes 10 and 11). As reported for the regulation of *rpoS* in *E. coli* (20), the results shown in Fig. 2A and C suggest that CRP activates *hapA* in *V. cholerae* by enhancing the transcription of *rpoS*. It is noteworthy that a conserved cAMP-CRP binding pentamer is located in the *V. cholerae* *rpoS* promoter. As cells progress into the stationary phase, an increase in the level of  $\sigma^S$  due to enhanced translation or protein stability could partially relieve the CRP dependency of *hapA*.

Comparison of *hapR* mRNA in isogenic *crp*<sup>+</sup> and *crp* strains, using quantitative real-time RT-PCR, suggested that CRP also enhances the transcription of *hapR* in cells collected at the growth deceleration phase (Fig. 2A and C). The link between CRP and HapR is interesting since it has been reported that *V. cholerae* quorum-sensing system 3 responds to an intracellular signal that acts through regulators LuxO and HapR (34). Our finding suggests that such internal signal could be cAMP.

We also noticed that cells grown to late stationary phase expressed lower levels of *hapA* mRNA (Fig. 1, compare lanes 2 and 3 with lanes 7 and 8). A phenomenon described as sigma factor competition has been found in *E. coli*, by which accumulation of  $\sigma^S$  lowers the expression of  $\sigma^{70}$ -dependent promoters (39, 48). Because transcription of *hapA* is subject to a dual regulation requiring both housekeeping and stationary-phase  $\sigma$  factors, we considered the possibility that the lower transcription of *hapA* in late stationary phase could be due to

reduced *hapR* expression. Strain AJB25 (*rpoS*) grown to late stationary phase expressed higher levels of a *hapR-lacZ* fusion than did the isogenic *rpoS*<sup>+</sup> strain, AJB26. However, the increase in expression of *hapR* in the *rpoS* mutant was lower than twofold, suggesting that additional factors could be involved.

We have previously reported that secretion of HA/protease is repressed by glucose (2). The presence of a region of dyad symmetry resembling a cAMP-CRP binding site in the *hapA* promoter suggested a carbon catabolite repression mechanism. However, although the *crp* exconjugant AJB3 expressed five times less  $\beta$ -galactosidase activity from the *hapA* promoter, it was still repressed by glucose (Fig. 3). This result demonstrates that repression of *hapA* transcription by glucose is not mediated by CRP. Accordingly, other sugars that might be under carbon catabolite repression themselves (i.e., galactose, mannose, maltose, sucrose, and glycerol) (16, 25) repressed *hapA* as efficiently as glucose did (data not shown). Repression of *hapA* by glucose strongly correlated with growth stimulation (Fig. 4A and B) and diminished the transcription of a *rpoS-lacZ* chromosomal fusion (Fig. 4C). By analogy to the regulation of  $\sigma^S$  in *E. coli* (20), glucose might also act on *rpoS* translation and  $\sigma^S$  stability. The above experiment clearly demonstrated that high cell density alone is not enough to activate *hapA* transcription, since glucose repression was accompanied by an increase in cell density.

When cultures of exponentially growing *V. cholerae* are induced to enter the stationary phase at a lower cell density, *hapA* transcription was detected at lower optical densities (Fig. 5A). Two factors could contribute to the expression of *hapA* at lower cell density: nutrient limitation could increase cAMP levels, leading to a CRP-mediated increase in HapR, and/or nutrient limitation promotes early entry in stationary phase, with accumulation of  $\sigma^S$ . Although these explanations are not mutually exclusive, the fact that *hapR* transcription was not significantly enhanced by nutrient limitation (Fig. 5B) suggests that entrance to the stationary phase is the predominant cause of early transcription of *hapA*. Taken together, our results show that *hapA* is transcribed in response to concurrent high cell density and nutrient limitation stimuli. It appears advantageous for *V. cholerae* to prevent *hapA* expression in populations entering the stationary phase at cell densities not supporting *hapR* expression, at which the formation of a biofilm is favored (47, 54).

In *E. coli*, transcription of *rpoS* and many *rpoS*-dependent genes is influenced by RelA activity (20). Nevertheless, strain AJB19 produced wild-type levels of HA/protease (Fig. 6). These results strongly suggest that the expression of *hapA* is not greatly affected by RelA activity. However, since only *relA spoT* double mutants entirely lack ppGpp (52), it is possible that the *relA* mutation in AJB19 does not reduce ppGpp levels enough to affect the expression of *hapA*.

Interestingly, pure His<sub>6</sub>-CRP or His<sub>6</sub>-HapR added separately or together did not bind the *hapA* promoter. This result indicates that HapR activates *hapA* by a mechanism different from that of its interaction with the *aphA* promoter. The *hapA* promoter does not contain the HapR recognition sequence present in *aphA* (26). The *hapA*<sup>+</sup> phenotypes of mutants AJB216 and AJB231 exclude the possibility of HapR controlling *hapA* through AphA (Fig. 6). Therefore, HapR could require additional factors to bind *hapA* or could control the

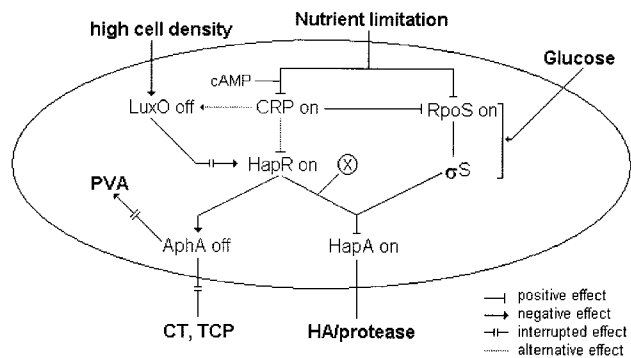


FIG. 8. Regulatory interactions involved in *hapA* transcription regulation. The *hapA* gene is transcribed in response to two concurrent environmental signals: high cell density and nutrient limitation. Nutrient limitation leads to entry of bacteria into the stationary phase, with enhanced transcription of *rpoS* and high levels of  $\sigma^S$ . At high cell density in the stationary phase, HapR is expressed and, in combination with an unidentified factor (X) and  $\sigma^S$ , activates the transcription of *hapA*. Glucose represses *hapA* transcription by inducing cells to resume exponential growth, blocking the pathway to accumulation of  $\sigma^S$ . The cAMP-CRP complex enhances the transcription of *hapA* by positively influencing the transcription of *rpoS* and *hapR*. CRP could increase HapR levels by acting on *luxO* or *hapR*. HapR represses the expression of *AphA*, required for the production of CT and TCP. In the absence of *AphA*, *pva*, encoding penicillin V amidase, is expressed.

expression of HA/protease through an unidentified regulator. In Fig. 8 we provide a model for the regulation of HA/protease expression in the context of other virulence factors, based on our current findings. The salient features this model are that transcription of *hapA* requires concurrent expression of *hapR* and *rpoS*, CRP enhances *hapA* transcription through *hapR* and *rpoS*, glucose represses *hapA* by inducing cells to resume exponential growth and lowering *rpoS* transcription, and HapR requires additional factors to activate *hapA*.

The transcriptional regulation of *hapA* reveal similarities and important differences compared to related metalloproteases from other members of the *Vibrionaceae*. A common property appears to be regulation by LuxR homologues such as HapR (*V. cholerae*), SmcR (*V. vulnificus*), or VanT (*V. anguillarum*) (10, 44). HA/protease differs in how its promoter integrates quorum-sensing and nutrient limitation stimuli. *V. vulnificus vvpE* elastase is transcribed from two promoters: promoter PL, constitutive throughout the logarithmic and stationary phases, and promote PS, dependent on SmcR, RpoS, and CRP (22). In contrast, *hapA* is not transcribed unless nutrient limitation induces cells to enter the stationary phase (Fig. 8). In *V. vulnificus*, CRP and SmcR coactivate elastase expression by binding to the PS promoter (23). CRP also binds to *V. harveyi* lux promoters to regulate luminescence (9). In *V. cholerae*, CRP activates *hapA* by increasing the transcription of *hapR* and *rpoS* (Fig. 8). CRP also increases the transcription of *luxR* in *V. fischeri* (12). These differences may play a role in enhancing the ability of different vibrios to adhere to and colonize their specific niches.

*V. cholerae* colonizes the distal small intestine, where most luminal nutrients (i.e., simple sugars) have already been absorbed (7). This mucin-rich environment could provide the signal to activate *hapA*. We have proposed that expression of HA/protease during infection could perturb the protective mu-

cus barrier, promote detachment, and spread the infection along the gastrointestinal tract. (3,4,45). Because the expression of HA/protease also requires high cell density, we would expect HA/protease effects to be more prominent in volunteers ingesting a high inoculum of *hapA*<sup>+</sup> live vaccines parallel with neutralization of stomach acidity.

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