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# Cyclic AMP and its Receptor Protein Negatively Regulate the Coordinate Expression of Cholera Toxin and Toxin-Coregulated Pilus in Vibrio Cholerae

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# Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*

**(gene regulation/environmental stimuli/pathogenesis)**

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**ABSTRACT** **Insertion mutations in two *Vibrio cholerae* genes, *cya* and *crp*, which encode adenylate cyclase and the cyclic AMP (cAMP) receptor protein (CRP), respectively, derepressed the expression of a chromosomal cholera toxin (CT) promoter–*lacZ* fusion at the nonpermissive temperature of 37°C. In the classical biotype strain O395, the *crp* mutation increased the production of both CT and toxin-coregulated pilus (TCP) *in vitro* under a variety of growth conditions not normally permissive for their expression. The most dramatic increase in CT and TCP was observed with the *crp* mutant in Luria–Bertani (LB) medium pH 8.5, at 30°C. El Tor biotype strains differ from classical strains in that they do not produce CT or TCP when grown in LB media. Incorporation of the *crp* mutation into El Tor strain C6706 permitted production of these proteins in LB medium pH 6.5, at 30°C. In the infant mouse cholera model, the *crp* mutation decreased colonization in both biotypes at least 100-fold relative to the wild-type strains. The data presented here suggest a model whereby cAMP–CRP negatively regulates the expression of CT and TCP in both classical and El Tor biotypes under certain environmental conditions and also influences pathogenesis by regulating other processes necessary for optimal growth *in vivo*.**

The human pathogen *Vibrio cholerae* O1 is a Gram-negative bacterium that colonizes the epithelium of the upper intestine and causes a severe diarrheal disease by secreting a potent ADP-ribosylating exotoxin. Cholera toxin (CT) is a multimeric protein composed of one A subunit and five B subunits encoded by the *ctxAB* operon (1). Coordinately expressed with CT is the toxin-coregulated pilus (TCP) (2), which plays a major role in the intestinal colonization by the bacterium. The transmembrane DNA-binding protein ToxR positively regulates the expression of CT, TCP, and accessory colonization factors (ACF) (3–5). Whereas activation of *ctx* is thought to occur by direct binding of ToxR to the promoter (5), ToxR controls the expression of the TCP and accessory colonization factor genes indirectly by activating the expression of another positive regulator, ToxT (6). Since the *toxT* gene is located within the TCP gene cluster, transcriptional readthrough from the *tcpA* promoter, the most proximal promoter in this operon, apparently also contributes to its expression (7). Although the mechanisms are not clearly understood, ToxT also plays a role in activating *ctx* expression (6).

The control of virulence gene expression in *V. cholerae* is strongly influenced by environmental stimuli. The function of such regulation may be to conserve resources under conditions that would not support a productive infection. One marked difference between the two epidemic biotypes of *V. cholerae* O1, classical and El Tor, is the way in which environmental stimuli exert control over the expression of ToxR-regulated genes. For example, with classical strains, the expression of *ctx*, *tcp*, and other genes in the ToxR regulon are maximal in Luria–Bertani (LB) medium pH 6.5, at 30°C in NaCl concentrations of approximately 66 mM (2, 3, 8). At 37°C, pH 8.5, or in higher or lower osmolarities, expression is reduced. In the case of El Tor strains, CT and TCP are not produced in LB media, but they can be induced by growth of the strains in a bicarbonate medium (AKI) without aeration followed by incubation with vigorous aeration (9). The mechanisms by which environmental stimuli exhibit control over the ToxR regulon in either biotype are presently unclear.

To elucidate the mechanisms by which stimuli from the environment control the expression of ToxR-regulated virulence genes, we constructed a chromosomal *ctx–lacZ* fusion in *V. cholerae* to screen for mutations in genes that alter this control. By screening for derepression of the fusion at the normally nonpermissive temperature of 37°C, we identified an insertion mutation in the *V. cholerae cya* gene, the structural gene for adenylate cyclase, which catalyzes the synthesis of cyclic AMP (cAMP). An insertion mutation in *crp*, the gene encoding the cAMP receptor protein (CRP) was subsequently engineered and was found to similarly derepress the expression of the *ctx–lacZ* fusion under certain environmental conditions. The cAMP–CRP system is a well-known global regulator of catabolite repression in enteric bacteria (for a review see ref. 10) and has recently received attention for its role in modulating virulence gene expression and pathogenesis in *Salmonella typhimurium* (11, 12). The results presented here indicate that in *V. cholerae* cAMP–CRP influences both the expression of ToxR-regulated virulence genes and pathogenesis.

## MATERIALS AND METHODS

**Bacterial Strains and Media.** *Escherichia coli* and *V. cholerae* strains were maintained at −70°C in LB medium (13) containing 30% (vol/vol) glycerol. The *V. cholerae* strains constructed in this study were derived from either the classical strain O395 (2) or its *lac*Δ derivative CG842 (14) and the El Tor strain C6706str2 (15): KSK218 (CG842 *ctx–lacZ*), KSK369 (KSK218 *crp*::*kan81*), KSK377 (O395 *crp*::*kan81*), KSK236 (KSK218 *toxR55)*, KSK374 (KSK369 *toxR55*), and KSK394 (C6706str2 *crp*::*kan81*). Antibiotics were used at the following concentrations in LB medium: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; kanamycin (Km), 45 μg/ml; chloramphenicol, 34 μg/ml for *E. coli* and 6 μg/ml for *V. cholerae*; streptomycin, 100 μg/ml except when selecting for loss of integrated plasmids in *V. cholerae*, where it was used at 1

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Abbreviations: CT, cholera toxin; TCP, toxin-coregulated pilus; CRP, cAMP receptor protein; Km, kanamycin; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; cfu, colony-forming units.

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mg/ml. In MacConkey agar (Difco), carbohydrates were used at 0.4% and antibiotics were used at concentrations ½ those listed above. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) was used in LB agar at 40 μg/ml.

**Construction of the *ctx–lacZ* Fusion.** The *ctx–lacZ* operon fusion was constructed as follows: A 542-bp *ctxA* fragment and a 492-bp *ctxB* fragment were amplified by PCR from *V. cholerae* O395 using oligonucleotide primers CTX1a (5′-GACTCGAATTCGCATGCGCAGTCAGGTG-GTCTTATG) and CTX1b (5′-GACTCGGATCCGAGCAT-TCCCACAACCCGG) and CTX2a (5′-GACTCGGATC-CTCTAGACCATGGATCAGTAATACTTGCGATG) and CTX2b (5′-GACTCGTCGACCGACTTTAGCTTCAG-TAAG), respectively. A promoterless *E. coli lacZ* gene and a chloramphenicol-resistance gene were inserted between the two *ctx* fragments in pKAS33, a derivative of pGP704 (8) containing the *rpsL* gene (16). The resulting fusion was introduced into CG842 by allelic exchange (16) to generate strain KSK218. Primer CTX1c (5′-CAGATTCTAGACCTC-CTGATG) was used with either LAC-1 (5′-GTCATAGCT-GTTTCCTGTGTG) or CTX2b to confirm the chromosomal location of the fusion.

**Tn*phoA* Mutagenesis.** Random insertion of Tn*phoA* into the chromosome of strain KSK218 was as previously described (2, 17) except that instead of screening for a $PhoA^+$ phenotype, dark blue colonies were identified on X-Gal plates at 37°C. To generate plasmid pKAS44, a 1-kb fragment was amplified from an internal portion of the *phoA* gene of Tn*phoA* using primers ALKP1 (5′-GACTCAGATCTGTAATTATGCCGAAGGT-GCGG) and ALKP2 (5′-GACTCGAGCTCCCCGGGTA-ACTCATCACCATCACTGCG) and was inserted into pKAS32 (16). Chromosomal capture plasmids were sequenced from either end of Tn*phoA* using primers TNPHOA-1 (5′-GCCGGGTGCAGTAATATCG) and IS50R-2 (5′-GAAGT-TATCATGAACGTTACC).

**Construction of the *crp*::*kan81* Mutation.** A 300-bp fragment from the *crp* gene of O395 was amplified using primers CR-1 (5′-GATAGCGGCCGCTGTGAAGTGGCTGAAAT-TTC and CR-3 (5′-GATAGCGGCCGCAGGTTCTGATC-CTCCAGCATC). A 19-bp deletion in the resulting fragment in plasmid pKAS69 was created by inverse PCR using primers CR-6 (5′-GATCGATATCAGGAACGCAAGGTCGC-CAAC and CR-7 (5′-GATCGATATCGCACAGACTCT-GCTGAATC). A Km-resistance gene was inserted into the 300-bp fragment at this position, and the resulting 1.3-kb fragment was inserted into pKAS32 to generate pKAS81. This mutation, designated *crp*::*kan81*, was then introduced into *V. cholerae* either by allelic exchange (16) or by CP-T1-mediated transduction (18).

**DNA Sequencing.** Cycle sequencing was performed using the Applied Biosystems Prism Dye System (Perkin–Elmer).

**β-Galactosidase and CT Assays.** Cultures were inoculated by dispersing colonies from plates into 1.5 ml of LB medium at an $OD_{600}$ of approximately 0.5. Fifty-fold dilutions of the cultures were then made into 4 ml of LB medium and incubated on a tube roller at the appropriate temperatures. β-Galactosidase assays (13) were carried out after 12 hr. The amount of protein in each reaction was determined by using the BCA (bicinchoninic acid) procedure (Pierce). GM1 ganglioside ELISA CT assays were carried out after 18 hr (19). The amount of protein in each cell pellet was determined as above. The data are representative results from at least two independent experiments.

**Gel Electrophoresis and Immunoblot Analysis.** Cell pellets from the CT assays were analyzed by SDS/12.5% polyacrylamide slab gel electrophoresis (PAGE) as described (2). Proteins were visualized either by staining with Coomassie blue or by transferring to nitrocellulose and probing with anti-TcpA antibody (20) using the ECL (enhanced chemiluminescence) detection system (Amersham).

**Colonization and $LD_{50}$ Assays.** The infant mouse competition assays were performed essentially as described (2). Three- to five-day-old suckling CD-1 mice (Charles River) were inoculated orally, and the total colony-forming units (cfu) was obtained from the small intestine of four mice at various times over a 24-hr period by plating intestinal homogenates on X-Gal plates with or without Km. For the two-strain experiments, the competitive index is defined as the difference between the input and output ratios of the strains. The $LD_{50}$ values were determined by oral challenge with various doses of viable bacteria. Four mice were used per dose and the results were analyzed after 36 hr. Prior to inoculation, all strains were grown in LB medium pH 6.5, at 30°C.

## RESULTS

**Isolation of Mutants Derepressed for *ctx* Expression by Using a Chromosomal *ctx–lacZ* Fusion.** A transcriptional fusion between the CT promoter and the *E. coli lacZ* gene was constructed in the *V. cholerae* chromosome to screen for insertion mutants with altered CT gene expression under different environmental conditions. The fusion was initially constructed on a plasmid by inserting a promoterless *lacZ* gene and a chloramphenicol-resistance gene between two *ctx* DNA fragments, and it was then transferred to the chromosome of strain CG842 by allelic exchange. The production of β-galactosidase in the resulting strain, KSK218, mirrored that of *ctx* expression both on X-Gal plates and in broth with respect to a variety of environmental conditions (see below), indicating that the fusion was properly regulated. The correct localization of the fusion into both genomic copies of *ctx* (21) was verified by PCR.

To isolate *V. cholerae* mutants derepressed for the expression of *ctx* at the normally nonpermissive temperature of 37°C, approximately 10,000 Tn*phoA* mutants of KSK218 were screened on X-Gal plates for colonies that appeared darker blue than the parent strain at this temperature. The mutations that produced the darkest blue colors were then backcrossed into KSK218 to verify that the observed phenotypes were due to the insertions. To efficiently backcross the Tn*phoA* insertions, we developed a method which we have termed "chromosomal capture." In this procedure, a plasmid carrying an internal portion of the *phoA* gene, pKAS44, was introduced into each insertion mutant, where it specifically integrated into the resident transposon. Chromosomal DNA was isolated from each integrant, digested with a restriction enzyme that does not cut within either Tn*phoA* or pKAS44, and ligated to favor the formation of monomolecular circles. After transformation of the DNA into S17-1 λ pir (22), selection for ampicillin and Km identified colonies harboring composite plasmids of pKAS44, Tn*phoA*, and chromosomal sequences flanking the transposon. To transfer the transposon insertions back into KSK218, the pKAS44 composite plasmids were ligated into the broad host range delivery plasmid pLAFR2 (23) and recovered in the streptomycin-resistant strain MC4100 (24). After selecting for loss of the pKAS44 portion of the composite plasmids on streptomycin and tetracycline, they were used to recombine the insertions into KSK218, using the IncP1 incompatibility system (25).

**Analysis of *V. cholerae cya* and *crp* Mutants.** After confirming the phenotypes of several of the strongest mutants identified above, the corresponding pKAS44 composite plasmids were sequenced to identify the transposon insertion sites. A data base BLAST search (26) with sequences from one mutant revealed that the transposon had inserted in a gene with very high homology to the adenylate cyclase (*cya*) genes from a number of different bacteria. Further analysis of this mutant showed that it indeed had phenotypes strikingly similar to those observed with strains that cannot synthesize cAMP (11, 27): a small colony size, slow growth rate, and an inability to ferment a variety of different carbohydrates. Furthermore, the

dark blue, small colony phenotype of the mutant on X-Gal plates was completely restored to wild type by the addition of 5 mM cAMP to the agar. A difficulty in working with the *cya* mutant was its propensity to revert to a wild-type phenotype at high frequency both on plates and in broth. Although we do not know the reason for this instability, one mechanism of *cya* suppression in *E. coli* is the formation of compensatory mutations in the *crp* gene (28). Thus, to avoid the instability problems of the *cya* mutant, we constructed a more stable mutation in the *V. cholerae crp* gene.

To construct the *crp* mutant, oligonucleotides designed from the *E. coli crp* sequence (29) were used to amplify an approximately 300-bp fragment from *V. cholerae* O395. Comparison of the sequence of the 300-bp fragment with that of the corresponding region of the *E. coli* gene (from amino acid 100 to 188) revealed 79% identity between the two at the nucleotide level and 94% identity at the amino acid level. A 19-bp deletion was introduced near the center of the 300-bp fragment, a Km-resistance gene was inserted at this position, and the resulting mutation was transferred into *V. cholerae*. The *crp* mutant strains produced small, slow growing colonies with a pleiotropic carbohydrate deficiency, phenotypes similar to those of the *cya* mutant described above except that they were stable on all media tested. As with the *cya* mutant, the KSK218 *crp* derivative KSK369 appeared dark blue on X-Gal plates at 37°C, indicating that *ctx* expression was similarly derepressed in this strain. The lighter blue color of the O395 *crp* mutant KSK377 on X-Gal plates indicates that the mutation reduced the expression of its endogenous *lacZ* gene. Despite the fact that *V. cholerae* does not ferment lactose, it is interesting that its *lacZ* gene still appears to be regulated by cAMP–CRP.

**The *crp* Mutant Is Derepressed for *ctx* Expression Under Nonpermissive Conditions.** The expression of the *ctx* operon is positively regulated by ToxR (4) and also controlled by environmental stimuli such as temperature, pH, and osmolarity (8). Fig. 1 compares the effects of temperature, pH, and a *toxR* mutation on the production of β-galactosidase in fusion strain KSK218 and its *crp* derivative KSK369. For classical strains, the optimal *in vitro* condition for the expression of *ctx* is LB medium pH 6.5, at 30°C. When grown under this condition, both KSK218 and its *crp* derivative KSK369 produced high levels of β-galactosidase. In pH 8.5 at 30°C, a condition not permissive for *ctx* expression, the production of β-galactosidase in KSK218 was reduced almost 10-fold relative to that at pH 6.5. In contrast, the *crp* mutant KSK369 produced essentially the same levels of β-galactosidase at both pH 6.5 and pH 8.5. This result indicates that the absence of functional CRP allows the strain to overcome the normal repression of *ctx* expression that occurs at pH 8.5. At 37°C, another condition not permissive for *ctx* expression, KSK218 produced 7- to 13-fold less β-galactosidase than at the optimal condition. Although the *crp* mutant showed a 4- to 5-fold increase in the production of β-galactosidase relative to KSK218 at 37°C, the strain did not completely overcome repression under this condition.

The introduction of a *toxR* mutation in KSK218 by insertion of plasmid pVM55 (8) into the chromosome resulted in a 20-fold reduction in β-galactosidase production in strain KSK236 relative to its *toxR*$^+$ parent in LB: pH 6.5 at 30°C, and, under the nonpermissive conditions, further decreased production approximately 2-fold. The *crp toxR* mutant KSK374 consistently showed a 2-fold increase in β-galactosidase production relative to KSK236 under all conditions examined. This result indicates that maximal derepression of *ctx* expression in the *crp* mutant depends on ToxR, yet suggests that cAMP–CRP functions independently of it.

**Increased Production of CT and TcpA under Nonpermissive Conditions by the *crp* Mutant.** Since CT and TCP are coordinately regulated, we examined their production in O395 and its *crp* derivative KSK377 under growth conditions similar to those used above. Autoagglutination is a property associated with expression of TCP in O395 that normally occurs *in vitro* at 30°C, pH 6.5, but not at pH 8.5 or at 37°C (2). Unlike O395, the *crp* mutants KSK369 and KSK377 both autoagglutinated at pH 6.5 and at pH 8.5 when grown at 30°C. Fig. 2 shows cultures of KSK369 and its parent KSK218 grown in LB, pH 8.5 at 30°C. The KSK218 culture (Fig. 2*A*) appears homogeneous, whereas in the KSK369 culture (Fig 2*B*) the cells aggregate and form a pellet on the bottom of the tube. SDS/PAGE analysis of total protein from O395 and KSK377 showed that the mutant indeed produced the 20.5-kDa major pilin protein TcpA at both pH 6.5 and pH 8.5 (Fig. 3, lanes 3 and 5). Similar levels of CT were also produced under both conditions. As expected, O395 produced TcpA at pH 6.5 but not at pH 8.5 (Fig. 3, lanes 2 and 4), and it produced 25-fold less CT at the higher pH. Although the *crp* mutant produced approximately twice as much CT as O395 at 37°C, neither

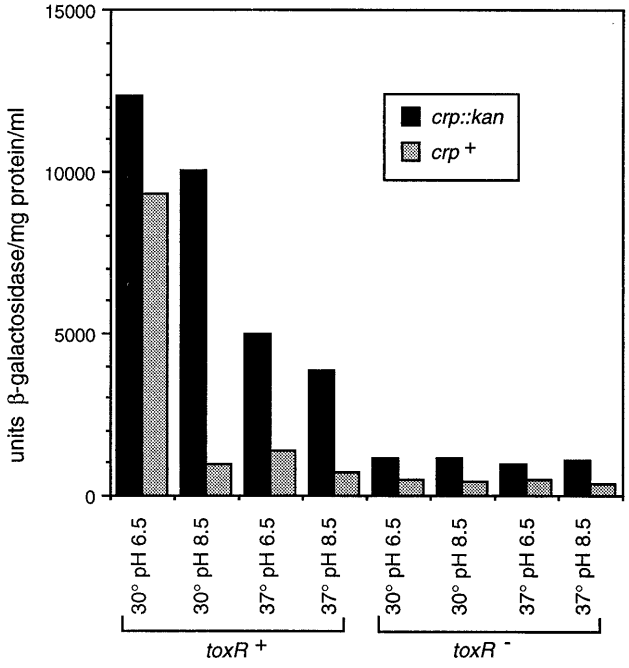


FIG. 1. β-Galactosidase production in the *ctx–lacZ* fusion strains. Left half: *toxR*$^+$ strains KSK369 (*crp*), black bars, and KSK218 (*crp*$^+$), grey bars. Right half: *toxR* strains KSK374 (*crp*), black bars, and KSK236 (*crp*$^+$), grey bars.

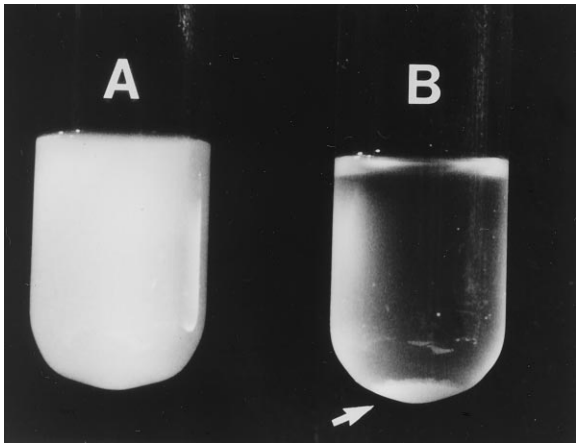


FIG. 2. Autoagglutination pattern of KSK218 and its *crp* derivative KSK369. Cultures of KSK218 (*A*) and KSK369 (*B*) were grown in LB medium, pH 8.5, at 30°C. Autoagglutinated bacteria are shown by the arrow.

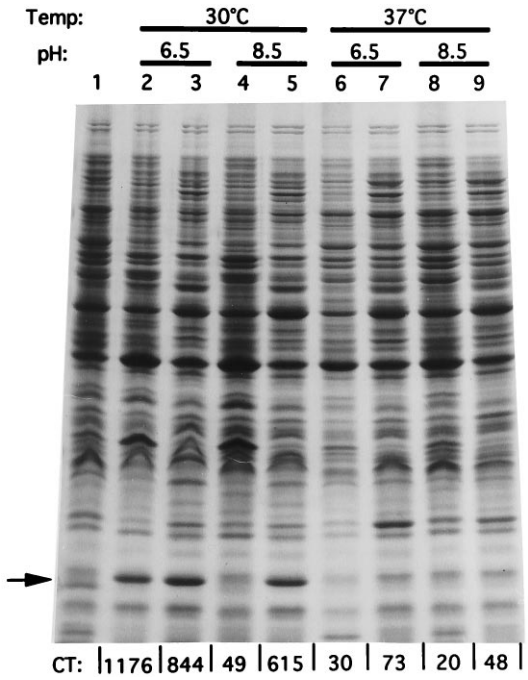


FIG. 3. TcpA and CT production in O395 and its *crp* derivative KSK377. Strains were grown in LB medium pH 6.5, at 30°C (lanes 2 and 3), pH 8.5, at 30°C (lanes 4 and 5), pH 6.5, at 37°C (lanes 6 and 7), and pH 8.5, at 37°C (lanes 8 and 9). For each strain, approximately 6 μg of total protein was subjected to SDS/PAGE and stained with Coomassie blue. CS2-1 (*tcpA*), lane 1; O395, even-numbered lanes; KSK377, odd-numbered lanes. TcpA is shown by the arrow. The amount of CT in each culture supernatant is expressed at the bottom as ng/mg of protein per ml.

strain produced TcpA at this temperature (Fig. 3, lanes 6–9). The pattern of derepression observed for *ctx* and *tcpA* with the *crp* mutant at pH 8.5, and to a lesser extent at 37°C, suggests that cAMP–CRP negatively regulates their expression under these conditions.

**Production of CT and TcpA by El Tor *crp* Mutants in LB Media.** Strains of the El Tor biotype require much more stringent growth conditions *in vitro* than classical strains to elicit the production of CT and TCP (9). The observation that the *crp* mutation increased the production of CT and TcpA in O395 under a number of normally nonpermissive growth conditions suggested to us that the mutation might have a similar effect in El Tor strains when grown in LB media, a condition normally repressive for CT and TcpA production in this biotype. As expected, El Tor strain C6706 did not produce TcpA detectable by Western blotting when grown in LB medium at 30°C and made less than 10 ng of CT per mg of protein per ml (Fig. 4, lanes 2 and 3). In contrast, the C6706 *crp* mutant KSK394 showed a strong TcpA signal when grown in LB medium, pH 6.5, at 30°C and produced approximately 25-fold more CT than its parental strain (Fig. 4, lane 4). The amount of CT produced by KSK394 was only 4-fold lower than that observed for O395. KSK394 also appeared to autoagglutinate in LB medium pH 6.5, at 30°C, but not to the extent normally observed with classical biotype strains. These results indicate that cAMP–CRP plays a role in the repression of CT and TcpA production in El Tor strains when grown in LB media. Interestingly, unlike the situation with classical strains, KSK394 did not produce detectable TcpA or CT at pH 8.5 (Fig. 4, lane 5), suggesting that even in the absence of a functional cAMP–CRP system, differences still exist in the way ToxR-regulated genes are controlled between the two biotypes.

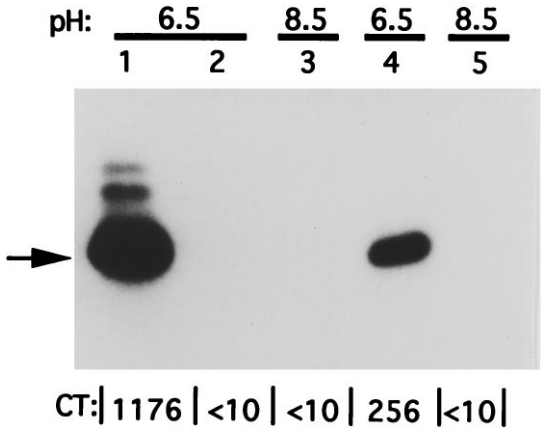


FIG. 4. TcpA and CT production in C6706 and its *crp* derivative KSK394. Strains were grown at 30°C in LB medium, pH 6.5 (lanes 1, 2, and 4), or pH 8.5 (lanes 3 and 5). For each strain, approximately 2 μg of total protein was subjected to SDS/PAGE, electroblotted to nitrocellulose paper, and probed with anti-TCP antiserum (20). O395, lane 1; C6706, lanes 2 and 3; KSK394, lanes 4 and 5. TcpA is shown by the arrow. The amount of CT in each culture supernatant is expressed at the bottom as ng/mg of protein per ml.

**Decreased Colonization of the *crp* Mutants in Infant Mice.** The effect of the *crp* mutation on virulence was assessed for O395 and its *crp* derivative KSK377 by determining the $LD_{50}$ values for each strain in the infant mouse cholera model. These values, $1 \times 10^6$ for O395 and $5 \times 10^8$ for KSK377, indicate that the *crp* mutation decreased the virulence of the strain approximately 500-fold.

Since KSK377 is not impaired for the production of either CT or TCP *in vitro*, it seemed unlikely that the attenuation of virulence observed with the *crp* mutant was due to a deficiency in the synthesis of factors important for colonization and virulence. To assess this, the colonization of KSK377 in infant mice was compared over a 24-hr period with both its O395 parent and a *tcpA* mutant, CS2-1 (30), that is defective for colonization (2). The results of these experiments, shown in Fig. 5, reveal striking differences in the colonization profiles of the three strains. The *tcpA* mutant was incapable of establish-

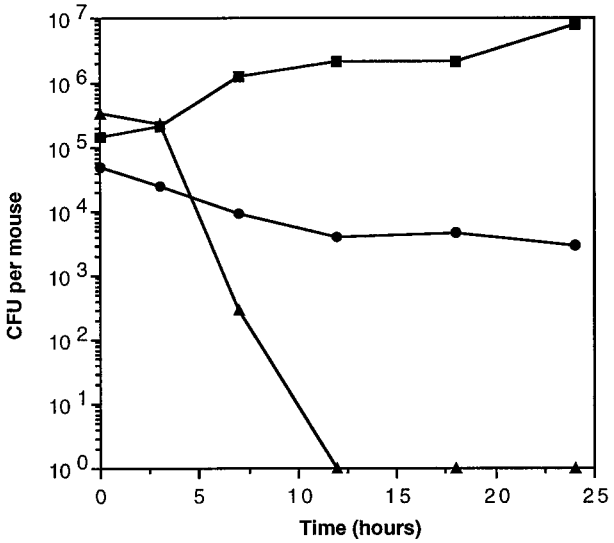


FIG. 5. Time course of colonization in the infant mouse cholera model. Twenty infant mice were orally inoculated with a mixture of strains O395, KSK377 (*crp*), and CS2-1 (*tcpA*). At various times over a 24-hr period, four mice were sacrificed and the total intestinal cfu per mouse for each strain was determined and averaged. ■, O395; ●, KSK377; ▲, CS2-1.

ing even a transient infection and was completely cleared from the intestine by 12 hr after inoculation. In contrast, KSK377 persisted in the intestine but consistently showed lower cfu per mouse compared with O395. At 12 hr the mutant was reduced approximately 100-fold relative to the wild type and by 24 hr it was reduced 1000-fold. When O395 and KSK377 were grown together *in vitro*, the mutant was reduced approximately 60-fold relative to the wild type by 12 hr. These results suggest that the decreased virulence of KSK377 is at least partially due to a defect in growth. The *in vitro* competitive index could not be calculated at late times in the growth curve, however, since the cfu for the mutant repeatedly decreased to undetectable levels. The mutant did not exhibit such a decrease when cultured alone *in vitro*, and the reasons for this phenomenon are presently under investigation. Consistent with the above hypothesis, the doubling time of KSK377 in LB at 37°C was increased approximately 30% relative to that of wild type, a value similar to that which has been observed with *E. coli crp* mutants (27).

The *crp* mutation also reduced the virulence of El Tor biotype strains. For C6706 and its *crp* derivative, KSK394, the $LD_{50}$ values were $5 \times 10^6$ and $>5 \times 10^8$, respectively. To compare the effect of the *crp* mutation on colonization between the two biotypes, the competitive indexes of KSK377 and KSK394 were each determined relative to their parents at 12 and 24 hr after inoculation. The competitive index of each mutant was between $10^{-1}$ and $10^{-2}$ at 12 hr and between $10^{-3}$ and $10^{-4}$ by 24 hr. The pattern of *in vitro* growth for the El Tor *crp* mutant was similar to that of the classical *crp* mutant, and the doubling time of the strain in LB medium at 37°C was also increased approximately 30% relative to wild type. Thus, with respect to growth and colonization, the behaviors of the classical and El Tor *crp* mutant strains are similar.

## DISCUSSION

The evidence presented here indicates that both adenylate cyclase and CRP influence the expression of ToxR-regulated virulence genes in response to environmental stimuli. Adenylate cyclase catalyzes the synthesis of cAMP, one of the most important and ubiquitous intracellular regulatory molecules. Together with its receptor protein, CRP, this system plays a central role in cellular metabolism in enteric bacteria by regulating the utilization of carbon and energy sources in the environment. In the presence of high intracellular levels of cAMP, binding of the nucleotide to CRP results in a conformational change in the protein that induces sequence-specific DNA binding. CRP functions as both a positive and negative effector of gene expression and influences many different cellular processes, including cell division and motility (10). Both cAMP and CRP also appear to have some individual regulatory roles. The similarities between the phenotypes of the *V. cholerae cya* and *crp* mutants described here and those of *E. coli* and *S. typhimurium* indicate that in *V. cholerae* the cAMP–CRP system functions in an analogous manner. However, one notable exception regards motility. *E. coli* and *S. typhimurium cya* and *crp* mutants do not synthesize flagella and are nonmotile (27, 31), whereas by phase-contrast and electron microscopy *V. cholerae cya* and *crp* mutants appear motile and produce flagella (data to be presented elsewhere). This finding is in contrast to a previous report that cAMP is indispensable for *V. cholerae* motility (32) and suggests that the synthesis of monotrichous flagella is regulated differently from that of peritrichous flagella.

A *crp* mutation in the classical biotype strain O395 and in the El Tor strain C6706 increased the production of both CT and TCP under growth conditions that are normally not permissive for their expression. In LB medium, pH 8.5, at 30°C, the O395 *crp* mutant produced essentially the same amounts of CT and TcpA as under the more optimal expression conditions. One interpretation of these results is that cAMP–CRP functions as a repressor of *ctx* and *tcpA* expression in LB medium, pH 8.5, at 30°C. Since repression of *ctx* was not completely alleviated in the *crp* mutant at 37°C, this result suggests that factors in addition to cAMP–CRP are involved in its repression at this temperature. In El Tor biotype strains, the absence of CRP permitted CT and TCP to be produced in LB medium, pH 6.5 at 30°C. Thus, in these strains cAMP–CRP is also involved in the repression of *ctx* and *tcpA* under certain growth conditions. The maximal level of CT produced in the C6706 *crp* mutant was still only one-third that of the O395 *crp* mutant and, unlike the situation with classical strains, no CT or TcpA was observed with the mutant in LB pH 8.5 at 30°C. Thus, fundamental differences over the control of the ToxR regulon exist between the two biotypes even in the absence of the cAMP–CRP system.

A putative consensus binding site for cAMP–CRP that overlaps the −35 sequence of the *tcpA* promoter has been identified in both classical and El Tor biotypes (33, 34). Although it is not yet known if CRP binds this site, its presence suggests a model for how cAMP–CRP may repress the expression of *tcpA* and *ctx* under particular growth conditions. If binding of the cAMP–CRP complex to this site occludes the −35 region of the *tcpA* promoter, cAMP–CRP could negatively regulate *tcpA* expression by preventing access of RNA polymerase. Although the more typical role for cAMP–CRP is as a positive regulator of transcription, there are a number of examples where cAMP–CRP negatively regulates gene expression. For example, in *E. coli*, binding of cAMP–CRP to the promoters of the *cya* and *gal* genes negatively regulates their expression by preventing access of RNA polymerase (35, 36). In *S. typhimurium,* cAMP–CRP negatively regulates the expression of the *spv* virulence operon (12), but the mechanisms involved in this regulation are not yet clear.

The coordinate effect of the *crp* mutation on *tcpA* and *ctx* expression could be explained if repression by cAMP–CRP at the *tcpA* promoter also influences the expression of the downstream regulatory gene *toxT*. ToxT has been shown to play a role in the activation of *ctx* (6), and although its expression is dependent upon ToxR (6), readthrough from the *tcpA* promoter contributes to its steady-state levels (7). Thus, in a *crp* mutant, derepression of *tcpA* would be expected to increase the expression of *toxT*, which would, in turn, increase *ctx* expression.

The above model is supported by a number of experiments that indicate that the influence of certain environmental conditions on the ToxR regulon is controlled at the level of *toxT* expression. For example, placing the *toxT* gene under a constitutive promoter in O395 alleviates the repression of *ctx* that occurs in LB medium pH 8.5 at 30°C (6) and similarly permits El Tor biotype strains to overcome the repression of *ctx* and *tcpA* expression that occurs in LB media (37). The striking similarity between the phenotypes of the *crp* mutants described here and those of the strains carrying the *toxT* constitutive constructs suggests that in the *crp* mutants the normal regulation of *toxT* expression has been altered. The model proposed here thus provides a mechanism for how *toxT* expression may be controlled by environmental conditions. The hypothesis that cAMP–CRP functions through ToxT rather than ToxR is also supported by the finding that in the *crp* mutants, derepression of *ctx* expression is observed even in the absence of ToxR. Many aspects of this model remain to be tested. For example, experiments are currently underway to determine if CRP binds to the site identified in the *tcpA* promoter and whether *toxT* message is increased in the *crp* mutants under the normally repressive growth conditions. We have also not ruled out the possibility that the effect of cAMP–CRP on the expression of *ctx* and *tcpA* is the result of its influence over some other aspect of cellular physiology.

The involvement of cAMP–CRP in the regulation of CT and TCP expression in *V. cholerae* suggests that carbon and energy

sources in the environment influence virulence gene expression. High intracellular levels of cAMP are indicative of growth on poor carbon sources, whereas lower levels are present in cells grown in more nutrient-rich environments. It is not yet clear, however, why cAMP–CRP would repress the ToxR regulon *in vitro* either in classical strains grown in LB medium, pH 8.5, at 30°C or in El Tor strains when grown in LB medium, pH 6.5, at 30°C. One possibility is that under these conditions the bacteria metabolize faster and exhaust their carbon sources more quickly than when grown in the more nearly optimal conditions for CT and TCP production. The down-regulation of virulence gene expression when cAMP levels are high may serve as a mechanism to limit the expression of virulence determinants in low-nutrient environments outside of the host and favor their expression in nutrient-rich environments such as the intestine. Previous *in vitro* studies have failed to demonstrate a relationship between cAMP levels and CT production in *V. cholerae* by varying the concentration of glucose in the growth media (38). It is possible that the levels of cAMP in these experiments were not sufficiently high to reduce CT production. Further work will be needed to elucidate how cAMP–CRP influences the expression of CT and TCP in *V. cholerae*.

The cAMP–CRP system regulates the expression of pilus and toxin genes in other bacteria. For example, in *E. coli*, Pap pili and heat-stable enterotoxin are both positively regulated by cAMP–CRP (39, 40). This mode of regulation would be expected to increase the expression of various functions to facilitate survival in low-nutrient environments. At least one other report exists of a pilus that is negatively regulated by cAMP–CRP, the I-sex pilus of *E. coli* (41). It has recently been shown in *V. cholerae* that TCP serves as the receptor for the phage CTX$\phi$ (42). That cAMP–CRP negatively regulates TCP expression suggests that the infectivity of the phage is controlled, at least in part, by intracellular levels of cAMP. In *E. coli* and *S. typhimurium*, lysogenization by temperate bacteriophages is influenced by intracellular cAMP levels.

The pathogenic potential of an organism is determined by many factors that influence its ability to survive and multiply within the host. The observation that the *crp* mutation in both classical and El Tor strains decreased their virulence in the infant mouse model is perhaps not surprising, considering the fact that the *crp* mutants grew slower *in vitro* than the wild-type strains. In *S. typhimurium*, *cya* and *crp* mutants show 10-fold derepression of the plasmid-borne *spv* virulence operon relative to wild-type strains upon entry into stationary phase, yet they are avirulent (11, 12). Although the molecular mechanisms responsible for these phenotypes are not yet understood, the lower growth rate of the mutant strains may be a contributing factor. Because of their avirulence, *S. typhimurium cya* and *crp* mutants have proven to be useful for the preparation of live oral vaccine derivatives and for the development of heterologous antigen-delivery systems (43). We are currently investigating whether *V. cholerae crp* mutants may have a similar utility for vaccine development. Live oral vaccine strains containing a *crp* mutation should colonize the intestine less avidly than the current strains and may produce fewer reactinogenic symptoms (44).

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