Cyclic AMP and Cyclic AMP-Receptor Protein are Required for Optimal Capsular Polysaccharide Expression

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Vibrio vulnificus causes fatal infections in susceptible individuals. Group 1 capsular polysaccharide (CPS) operon is responsible for CPS expression, which plays an essential role in the pathogenesis of this pathogen. Cyclic AMP (cAMP) and cAMP receptor protein (Crp) complex, which responds to glucose availability and functions as a global regulator, has been known to affect CPS production in this pathogen. This study was undertaken to experimentally verify whether cAMP-Crp directly or indirectly affects CPS production. A mutation in cyaA encoding adenylate cyclase, which is required for cAMP biosynthesis, inhibited V. vulnificus growth and changed opaque colonies to translucent colonies, and these changes were recovered by complementing cyaA or by adding exogenous cAMP. A mutation in crp encoding Crp also inhibited V. vulnificus growth and changed opaque colonies to translucent colonies, and these changes were recovered by complementing crp. Moreover, the crp or cyaA mutation decreased the susceptibility of V. vulnificus against NaOCl. The crp mutation reduced the transcription levels of group 1 CPS operon on a per cell basis. Glucose addition in the absence of Crp stimulated V. vulnificus growth, changed translucent colonies to opaque colonies, and increased the transcription levels of group 1 CPS operon. These results indicate that cAMP or Crp is indirectly involved in optimal CPS production by positively affecting metabolism or V. vulnificus growth rather than by directly controlling the expression of group 1 CPS operon.

Key Words: Vibrio vulnificus, Glucose, Adenylate cyclase, Cyclic AMP-receptor protein, Capsular polysaccharide

INTRODUCTION

Vibrio vulnificus is a gram-negative halophilic bacterium that is capable of causing life-threatening septicemia and necrotizing wound infections that can progress rapidly and have high mortality rates. Several established and potential virulence factors have reported to play important roles in the pathogenesis of V. vulnificus infections, such as, capsular polysaccharides (CPS), iron acquisition systems, flagella or motility, pili, and cytotoxins (1). These factors appear to be under the control of global regulators, such as, cyclic AMP (cAMP) and cAMP receptor protein (Crp) complex (2–4), the quorum sensing master regulator SmcR (5), and the alternative sigma factor of RNA polymerase RpoS (6).

In V. vulnificus, CPS production is clearly a prerequisite for virulence and correlates with lethality in mice (7–9), complement-mediated lysis (10), cytokine induction (11),...
and resistance to phagocytosis (12). CPS is a protective antigen in mice (13, 14). CPS expression is correlated with colony opacity (7, 9). Accordingly, opaque and translucent phenotypes remain the most reliable predictors of virulence despite the presence of other virulence factors (15–18).

Several genetic loci are involved in CPS biosynthesis, polymerization, and transport in \textit{V. vulnificus} (19–21). Of these loci, group 1 CPS operon consists of a partial open reading frame (ORF) lacking a start codon, \textit{wza}, \textit{wzb} and \textit{wzc} encoding outer membrane proteins involved in CPS transport, and several genes required for CPS biosynthesis (20). \textit{V. vulnificus} strains exhibit great diversity in CPS carbohydrate composition (22, 23) and genetic variation in the group 1 CPS operon (24).

The regulation of CPS production has not been extensively studied in \textit{V. vulnificus}. Previous studies have reported that CPS production is growth phase- and temperature-dependent (25), and that reversible phase variation from opaque to translucent colonies occurs despite no mutation is present in group 1 CPS operon (24) and the rate of phase variation is considerably condition-dependent (26). These findings imply that CPS production can be regulated in response to environmental signals. In most bacteria, cAMP increases in response to glucose deprivation by the action of adenylate cyclase and binds to Crp, and cAMP-Crp complex is involved in catabolite repression (27). Crp or cAMP is also involved in the expressions of a variety of genes associated with virulence in most bacteria including \textit{V. vulnificus} (2–4, 28–30). It has been known that a mutation in \textit{cyaA} encoding adenylate cyclase, which is required for cAMP synthesis, or in \textit{crp} encoding Crp affects CPS expression and changes opaque \textit{V. vulnificus} colonies to translucent colonies in \textit{V. vulnificus} (2–4). However, it remains unknown whether cAMP or Crp directly or indirectly affects CPS production. Therefore, this study was undertaken to determine the effect of glucose, cAMP or Crp on the expression of group 1 CPS operon. For this study, we constructed \textit{cya} and \textit{crp} mutants and \textit{lacZ}-fused \textit{wza} transcription reporter strains. The \textit{wza} transcription reporter is also the transcription reporter of group 1 CPS operon because group 1 CPS operon is expressed as a single transcript and a partial ORF upstream of \textit{wza} does not have a start codon. In addition, \textit{V. vulnificus} strains with thick capsule are known to be more susceptible to sodium hypochlorite (NaOCl) than \textit{V. vulnificus} strains with thin capsule (31). Therefore, in this study, the susceptibility of \textit{V. vulnificus} to NaOCl was determined in the presence or absence of Crp.

### MATERIALS AND METHODS

#### Bacterial strains, plasmids, primers, media, and reagents

The \textit{V. vulnificus} and \textit{Escherichia coli} strains, plasmids, and PCR primers used in this study are listed in Table 1. For cultivating \textit{V. vulnificus} strains, 2.0% NaCl was additionally added into Heart Infusion (HI; BD, Franklin Lakes, NJ, USA) agar or broth. Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS, BD) agar was used to select \textit{V. vulnificus} strains from \textit{E. coli} strains. LB medium was used for cultivating \textit{E. coli} strains. To observe the effect of glucose, 100 mM N-trismethyl-2-aminoethanesulfonic acid (TES) as a pH buffering agent (32) was added to HI broths and agars (TES-HI broths and agars). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

#### Deletion mutation and in trans complementation of genes

RC398 containing an \textit{in frame} deletion of \textit{wza} was constructed as follows. Two pairs of the PCR primers \textit{wza-up-1/wza-up-2} and \textit{wza-down-1/wza-down-2} were used for amplifying the 5’ and 3’ fragments of \textit{wza}, respectively. The resulting PCR products were used as templates for second PCR amplification using the PCR primers \textit{wza-up-1} with a \textit{BglII} overhang and \textit{wza-down-2} with a \textit{XbaI} overhang. The \textit{BglII-XbaI} fragment containing deleted \textit{wza} was cloned into pDM4 (33), and the resulting pRC348 was transformed into \textit{E. coli} SY327 \textit{λpir} and SM10 \textit{λpir} (34), and subsequently introduced to M06-24/O by conjugation.

The constructions of CMM710 containing deleted \textit{crp
Table 1. Bacterial strains, plasmids and primers used in this study

| Strains/plasmids/primers | Relative characteristics and sequences | References or sources |
|--------------------------|----------------------------------------|----------------------|
| **Vibrio vulnificus**    |                                        |                      |
| M06-24/O                 | Opaque, highly virulent clinical isolate | 22                   |
| RC398                    | M06-24/O with in frame deleted wza     | This study           |
| CMM710                   | M06-24/O with mutated crp              | 3                    |
| CMM714                   | CMM710 with in trans complemented crp  | 3                    |
| RC386                    | M06-24/O with mutated cyaA             | This study           |
| RC390                    | RC386 with in trans complemented cyaA  | This study           |
| CMM2101                  | M06-24/O with mutated lacZ             | 32                   |
| RC100                    | CMM2101 with mutated crp               | This study           |
| RC342                    | CMM2101 with P<sub>reg</sub>-lacZ fusion | This study         |
| RC344                    | RC100 with P<sub>reg</sub>-lacZ fusion | This study           |
| RC360                    | RC122 with in trans complemented crp   | This study           |
| **Escherichia coli**     |                                        |                      |
| SY327 λpir               | Host for suicide vector                | 34                   |
| SM10 λpir                | Conjugation donor                      | 34                   |
| **Plasmids**             |                                        |                      |
| pDM4                     | Suicide vector with R6K origin         | 33                   |
| pRK20132                 | Conjugation helper vector              | 35                   |
| pQF5                     | IncP lacZ transcriptional fusion vector | 36                   |
| pLAFR3II                 | pLAFR3 with bla inserted at the cos site | 32                   |
| pRC348                   | pDM4 with 1.547-kb BglII-XbaI fragment containing in-frame deleted wza | This study |
| pRC316                   | pDM4 with 1.63-kb XbaI-SmaI fragment containing in-frame deleted cyaA | This study |
| pRC318                   | pLAFR3II with 3.16-kb BamHI-EcoRI fragment containing wild-type cyaA | This study |
| pRC298                   | pQF52 with 886-bp BamHI-HindIII fragment containing the promoter region of wza | This study |
| pRC302                   | pDM4 with BglII-ScaI fragment containing P<sub>reg</sub>-lacZ fusion | This study |
| pCMM712                  | pLAFR3 with 6-kb HindIII fragment containing wild-type crp | 3 |
| **Primers**              |                                        |                      |
| wza-up-1                 | 5’-gaagatcttcagttggttagtagactaagc-3’ | This study           |
| wza-up-2                 | 5’-taatcatcttcatactgagttctcaacatagtaagc-3’ | This study |
| wza-down-1               | 5’-gaaatccacttctcagttggttagtagactaagc-3’ | This study |
| wza-down-2               | 5’-ctgtagaacaactgtagaagatcgtctttagagtaagc-3’ | This study |
| cyaA-up-1                | 5’-gctcgaagctcaagcgccgctcaacgctaatag-3’ | This study |
| cyaA-up-2                | 5’-gctcgaagctcaagcgccgctcaacgctaatag-3’ | This study |
| cyaA-down-1              | 5’-ctgtagaacaactgtagaagatcgtctttagagtaagc-3’ | This study |
| cyaA-down-2              | 5’-ctgtagaacaactgtagaagatcgtctttagagtaagc-3’ | This study |
| cyaA-comp-1              | 5’-gctcgaagctcaagcgccgctcaacgctaatag-3’ | This study |
| cyaA-comp-2              | 5’-gctcgaagctcaagcgccgctcaacgctaatag-3’ | This study |
| 1-CPS-rep-F              | 5’-tgctcgaagctcaagcgccgctcaacgctaatag-3’ | This study |
| 1-CPS-rep-R              | 5’-gctcgaagctcaagcgccgctcaacgctaatag-3’ | This study |
and CMM714 containing in trans complemented crp were performed as previously described (3). RC386 containing a deletion of cyaA was constructed as follows. Two pairs of PCR primers cyaA-up-1/cyaA-up-2 and cyaA-down-1/cyaA-down-2 were used for amplifying the 5′ and 3′ ends of cyaA, respectively. The resulting PCR products were used as templates for second PCR amplification using the PCR primers, cyaA-up-1 with an XbaI overhang and cyaA-down-2 with a SmaI overhang. The XbaI-SmaI fragment containing deleted cyaA was cloned into pDM4 (33), and the resulting plasmid (pRC316) was transformed into E. coli SY327 λpir and SM10 λpir (34), and subsequently introduced to M06-24/O (22) by conjugation. For restoring wild-type cyaA in RC386, a 3.16-kb BamHI-EcoRI fragment encompassing wild-type cyaA was amplified using the PCR primers, cyaA-comp-1 with a BamHI overhang and cyaA-comp-2 with an EcoRI overhang. The PCR product was subcloned into pLAFR3II (35), and the resulting plasmid pRC318 was introduced into RC386 by triparental mating using pRK2013 (32).

Construction of lacZ-fused wza transcription reporters

RC342 containing a lacZ-fused wza transcription reporter was constructed as follows. An 886-bp fragment extending from -736 to +150 bp from the translation start codon of wza (20) was amplified using the PCR primers, 1-CPS-rep-F with a BamHI overhang and 1-CPS-rep-R with a HindIII overhang. The resulting PCR fragment was subcloned into pQF52 (36). From the resulting plasmid pRC298, a BamHI-ScaI fragment containing the P_wza::lacZ construct was subcloned into pDM4 (33). The resulting plasmid pRC302 was transformed into E. coli SY327 λpir and SM10 λpir (34), and transferred into CMM2101 by conjugation. For restoring wild-type crp in RC344, plasmid pCMM712 containing wild-type crp was introduced into RC344 via triparental mating using pRK2013, and the resulting strain was named RC360.

Observation of colony morphology

The colony morphologies of V. vulnificus strains were observed on HI agar plates. V. vulnificus strains cultured overnight at 37°C in HI broths were spread on the surface of HI agar plates using an ordinary loop, and plates were incubated for 24 h at 37°C and then photographed. When necessary, cAMP was added to a final concentration of 100 or 500 μM into HI agars and glucose was added to a final concentration of 0.25% into TES-HI agars prior to solidification.

Chlorine resistance assay

For V. vulnificus strains, chlorine resistance was determined as previously described (31). Newly purchased NaOCl was used, and the sealed containers were not opened until immediately prior to dilution to obtain a concentration of 1.5 μg/ml in phosphate-buffered saline (PBS, pH 7.2). A representative colony from each strain was picked up with an ordinary loop and cultured in HI broth at 37°C for 6 h. Approximately 10^5 mid-exponential-phase cells were exposed to either PBS or 1.5 μg/ml NaOCl for 5 min, which was followed by dilution and plating.

Measurements of bacterial growth and β-galactosidase activity

V. vulnificus strains were inoculated into HI broths at a bacterial density of 5 × 10^6 cells/ml, and cultured with vigorous shaking (200 rpm) at 37°C for 24 h. Culture aliquots were removed at appropriate times to measure bacterial growth and gene transcription levels. Bacterial growth levels were measured by the optical densities of culture aliquots at 600 nm (OD_600). Gene transcription levels were monitored by measuring β-galactosidase activities on a per cell basis in culture aliquots, as previous described (37).

RESULTS

Effect of wza mutation on CPS production

Whether group 1 CPS operon, especially its first gene
wza, is essential for CPS production was determined by culturing M06-24/O containing wild-type wza and RC398 containing mutated wza on HI agar plates and examining their colony morphologies. M06-24/O formed typical opaque colonies, whereas RC398 produced translucent colonies (Fig. 1A & 1B).

**Effects of cyaA and crp mutation on CPS production**

The effect of a mutation in cya or crp on CPS production was determined by culturing *V. vulnificus* strains on HI agar plates. M06-24/O containing wild-type cya and crp formed relatively larger and typical opaque colonies, whereas RC386 containing mutated cya and CMM710 containing mutated crp formed relatively smaller translucent colonies. These changes in RC386 were prevented in RC390 containing in trans complemented cya and those in CMM710 were prevented in CMM714 containing in trans complemented crp (Figs. 1C to 1H). Translucent colonies in RC386 were changed to opaque colonies by the addition of exogenous cAMP (Figs. 1I, 1J & 1K).

**Effects of cyaA and crp mutation on chlorine resistance**

For the relative comparison of CPS production among *V. vulnificus* strains, resistance to chlorine disinfectant was tested (Fig. 2). The differences between the PBS- and NaOCl-treated groups were significant for all strains (*p* < 0.05, Two-way ANOVA). In the PBS-treated group, there was no difference among strains. In the NaOCl-treated group, M06-24/O containing wild-type cya and crp was almost killed, whereas RC386 containing mutated cya and CMM710 containing mutated crp survived more. The mean viable cell counts for RC386 and CMM710 were 1 to 2 logs higher than those for M06-24/O or those for RC390 and CMM714.
Effect of \( crp \) mutation on the transcription of group 1 CPS operon

The effect of a mutation in \( crp \) on the expression of group 1 CPS operon was examined by culturing the \( P_{\text{wza}}::\text{lacZ} \) transcription reporter strains in HI broths, and by comparing their growth and \( \text{wza} \) transcription levels. The growth of RC344 containing mutated \( crp \) was significantly lowered compared to those of RC342 containing wild-type \( crp \) and RC360 containing complemented \( crp \) (\( p < 0.05 \), Student's \( t \)-test) (Fig. 3A). The \( \text{wza} \) transcription levels on a per cell basis began to be steeply increased from the early growth phase and this increase was slightly decreased during the late growth phase (Fig. 3B). The growth and \( \text{wza} \) transcription levels of RC344 containing mutated \( crp \) were significantly lower than those of RC342 containing wild-type \( crp \) or those of RC360 containing in trans complemented \( crp \) (\( p < 0.05 \), Student's \( t \)-test).

Effect of glucose on CPS expression and the transcription of group 1 CPS operon

The effect of glucose on CPS production was determined by culturing \( V. \text{vulnificus} \) strains on TES-HI agar plates containing 0 and 0.25% glucose. The addition of glucose had no noticeable effect on the colony opacity of M06-24/O containing wild-type \( crp \) and CMM714 containing in trans complemented \( crp \) (data not shown). In contrast, the addition of glucose changed translucent colonies changed to opaque colonies in CMM710 with mutated \( crp \) (Fig. 4). The addition
of larger amount of glucose (especially >0.3%) changed opaque colonies to translucent colonies in all *V. vulnificus* strains including CMM710 (data not shown). In M06-24/O and CMM714, opaque colonies were also changed to translucent colonies when the pH of HI media was adjusted to below 6.5 with no glucose addition (data not shown). Accordingly, this might be due to the effect of low pH (pH < 6.5), which resulted from glucose catabolism.

The effect of glucose on the expression of group 1 CPS operon was examined by culturing the P<sub>wza</sub>::lacZ transcription reporter strains in TES-HI broths containing 0.0 and 0.25% glucose, and by comparing its growth and wza transcription levels. The addition of glucose had no significant effect on the growth and wza transcription levels of RC342 containing wild-type *crp* and RC360 containing *in trans* complemented *crp* (data not shown). In contrast, the addition of glucose significantly increased the growth and wza transcription levels of RC344 containing mutated *crp* (*p < 0.05*) (Figs. 5A and 5B). The addition of larger amount of glucose (especially >0.3%) decreased wza expression levels in all *V. vulnificus* strains including CMM710 (data not shown).

**DISCUSSION**

In *V. vulnificus*, cAMP or Crp has found to be involved in the expression of several virulence factors including CPS production, motility, the productions of hemolysin and metalloprotease, the expressions of iron-uptake systems, cytotoxicity, and lethality in mice (2–4). Furthermore, Crp has been shown to regulate the expressions of the hemolysin *vvhBA* (28), the metalloprotease *vvpE* (29, 38), the vulnibactin receptor *vuuA* (3), the heme receptor *hupA* (30) and the aerobactin receptor *iutA* (39) genes by directly binding to the Crp binding sites in the regulatory regions of these genes. However, the mechanistic details of the involvements of Crp in CPS production and motility remain unknown.

As observed in a previous study (20), the present study also shows that opaque colonies changed to translucent colonies by only an *in frame* deletion mutation of *wza*, which is one of several genes belonging to group 1 CPS operon and involved in extracellular CPS transport. This finding indicates that group 1 CPS operon plays an important role in CPS expression in *V. vulnificus*. However, it has also been reported that opaque colonies can be reversibly changed to translucent colonies with no mutation in group 1 CPS.
operon (24).

In some encapsulated pathogens, CPS production is dependent on environmental factors such as pH, nutritional levels, metal cation availability, and growth phase (40–44). These findings indicate that bacteria can respond to environmental signals to regulate CPS production, and thereby, increase virulence and enhance survival in different environments or hosts. Similarly, *V. vulnificus* is likely to respond to environmental signals to regulate CPS production.

In *V. vulnificus*, CPS production varies with incubation temperature and growth phase, as determined by semiquantitative assays using CPS-specific monoclonal antibody (25). CPS production peaks during the logarithmic growth phase and declines as cells reach the stationary growth phase. The present study also shows a growth phase-dependent group 1 CPS operon expression at the transcription level: its expression levels increased more rapidly during the early growth phase than during the late growth phase. This growth phase-dependency of CPS operon expression might be related to increased quorum sensing activity or to the deprivation of nutrients. In fact, a mutation in *smcR* encoding the master quorum sensing regulator SmcR has been reported to affect colony opacity (5). However, a previous study also demonstrated that phase variation from opaque to translucent colonies is affected by RpoS, but not by quorum sensing (26). Overall, the effects of RpoS and of SmcR on the expression of group 1 CPS operon need to be directly determined by more coherent studies.

In the present study, a *cya* mutation changed opaque colonies to translucent colonies, and this change was prevented by the addition of exogenous cAMP. A *crp* mutation changed opaque colonies to translucent colonies, and reduced the expression of group 1 CPS operon on a per cell basis, especially during the late growth phase. In addition, the *cya* or *crp* mutation caused *V. vulnificus* to be more resistant to NaOCl. These changes induced by the *cya* or *crp* mutation was prevented by *in trans* complementing *cya* or *crp*. However, on transmission electron micrograph, we could not find a remarkable difference in the thickness of CPS layer among strains irrespective of the *cya* or *crp* mutation (data not shown). Moreover, in sequence analysis, putative Crp-binding sites showing a high similarity to the Crp-binding consensus (TGTGA-(N<sub>6</sub>)-TCACA) were not found in the regulatory region of group 1 CPS operon (20, 45). Overall, these indicate that Crp is not a major or an essential regulator for CPS production but required for optimal or full CPS production, and that cAMP or Crp affects CPS production and group 1 CPS operon expression in an indirect manner.

Glucose likely is a preferred carbon or energy source in *V. vulnificus*, and cAMP or Crp is essentially required for the utilization of alternative carbon or energy sources and to improve the growth and survival of *V. vulnificus* under glucose-deficient conditions. The HI broth used in this study contains only an undefined low level of glucose. Accordingly, glucose contained in the HI broth would be consumed during the early growth phase, and Crp or cAMP would be induced in response to glucose deprivation during the late growth phase. The absence of cAMP or Crp under glucose-deficient conditions severely impairs metabolism and eventually inhibits *V. vulnificus* growth, as also observed in previous studies (2–4, 38, 39). This impairment of metabolism or growth of *V. vulnificus* in the absence of cAMP or Crp may affect CPS expression and the expression of group 1 CPS operon. In the present study, the addition of glucose into HI media recovered *V. vulnificus* growth, changed from translucent colonies to opaque colonies, and increased the transcription levels of *wza* in the absence of Crp as shown in Fig. 4 and 5. However, these findings were not observed in the presence of Crp (data not shown). Both low pH (pH < 6.5) itself or low pH which is caused by metabolizing excessive glucose (>0.3%) also inhibited *V. vulnificus* growth and changed opaque colonies to translucent colonies (data not shown). In *Streptococcus pneumoniae*, excessive glucose also inhibited CPS production by inhibiting bacterial growth via secondary metabolites such as lactic acid (44). Accordingly, optimal growth (metabolism) is a prerequisite for optimal CPS production, and cAMP or Crp is likely to be indirectly involved in CPS production and group 1 CPS operon expression by affecting metabolism or *V. vulnificus* growth.
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