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Original Article

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),

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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 *V. vulnificus* (19~21). Of these loci, group 1 CPS operon consists of a partial open reading frame (ORF) lacking a start codon, *wza*, *wzb* and *wzc* encoding outer membrane proteins involved in CPS transport, and several genes required for CPS biosynthesis (20). *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 *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 *V. vulnificus* (2~4, 28~30). It has been known that a mutation in *cyaA* encoding adenylate cyclase, which is required for cAMP synthesis, or in *crp* encoding Crp affects CPS expression and changes opaque *V. vulnificus* colonies to translucent colonies in *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 *cya* and *crp* mutants and *lacZ*-fused *wza* transcription reporter strains. The *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 *wza* does not have a start codon. In addition, *V. vulnificus* strains with thick capsule are known to be more susceptible to sodium hypochlorite (NaOCl) than *V. vulnificus* strains with thin capsule (31). Therefore, in this study, the susceptibility of *V. vulnificus* to NaOCl was determined in the presence or absence of Crp.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, media, and reagents

The *V. vulnificus* and *Escherichia coli* strains, plasmids, and PCR primers used in this study are listed in Table 1. For cultivating *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 *V. vulnificus* strains from *E. coli* strains. LB medium was used for cultivating *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 *in frame* deletion of *wza* was constructed as follows. Two pairs of the PCR primers wza-up-1/wza-up-2 and wza-down-1/wza-down-2 were used for amplifying the 5' and 3' fragments of *wza*, respectively. The resulting PCR products were used as templates for second PCR amplification using the PCR primers wza-up-1 with a *Bgl*II overhang and wza-down-2 with a *Xba*I overhang. The *Bgl*II-*Xba*I fragment containing deleted *wza* was cloned into pDM4 (33), and the resulting pRC348 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (34), and subsequently introduced to M06-24/O by conjugation.

The constructions of CMM710 containing deleted *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_{wza}::*lacZ* fusion	This study
RC344	RC100 with P_{wza}::*lacZ* fusion	This study
RC360	RC122 with *in trans* complemented *crp*	This study
Escherichia coli		
SY327 λ*pir*	Host for suicide vector	34
SM10 λ*pir*	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 *Bgl*II-*Xba*I fragment containing *in-frame* deleted *wza*	This study
pRC316	pDM4 with 1.63-kb *Xba*I-*Sma*I fragment containing *in-frame* deleted *cyaA*	This study
pRC318	pLAFR3II with 3.16-kb *Bam*HI-*Eco*RI fragment containing wild-type *cyaA*	This study
pRC298	pQF52 with 886-bp *Bam*HI-*Hin*dIII fragment containing the promoter region of *wza*	This study
pRC302	pDM4 with *Bgl*II-*Sca*I fragment containing P_{wza}::*lacZ* fusion	This study
pCMM712	pLAFR3 with 6-kb *Hin*dIII fragment containing wild-type *crp*	3
Primers		
wza-up-1	5'-gaagatcttcaagtgttggcttagagactaaagc-3'	This study
wza-up-2	5'-taaatcatcttacataggtattcaacatagttaaaggc-3'	This study
wza-down-1	5'-tgaatacctatgtaagatgatttaaaagcggtcctatgg-3'	This study
wza-down-2	5'-gctctagaaaacatgagtaatgcctattgaattattg-3'	This study
cyaA-up-1	5'-gctctagaagccagcggccgcgagaatgatc-3'	This study
cyaA-up-2	5'-cgctttggacatcttctgactttgcaaatccataagcgccag-3'	This study
cyaA-down-1	5'-gatttgcaaagtcagaagatgtccaaagcggtcaacgtatag-3'	This study
cyaA-down-2	5'-tcccccgggtgcctactgtgattgctcagattgttg-3'	This study
cyaA-comp-1	5'-cgggatcctgcacgcccttccagcattgc-3'	This study
cyaA-comp-2	5'-ggaattcgcgtagctatcgttaagccattaag-3'	This study
1-CPS-rep-F	5'-cgggatccactgctgttgccgctactgctg-3'	This study
1-CPS-rep-R	5'-cccaagcttggcaagcaaggcaggcgctag-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 *Xba*I overhang and cyaA-down-2 with a *Sma*I overhang. The *Xba*I-*Sma*I fragment containing deleted *cyaA* was cloned into pDM4 (33), and the resulting plasmid (pRC316) was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (34), and subsequently introduced to M06-24/O (22) by conjugation. For restoring wild-type *cyaA* in RC386, a 3.16-kb *Bam*HI-*Eco*RI fragment encompassing wild-type *cyaA* was amplified using the PCR primers, cyaA-comp-1 with a *Bam*HI overhang and cyaA-comp-2 with an *Eco*RI 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 *Bam*HI overhang and 1-CPS-rep-R with a *Hin*dIII overhang. The resulting PCR fragment was subcloned into pQF52 (36). From the resulting plasmid pRC298, a *Bam*HI-*Sca*I fragment containing the P_{wza}::*lacZ* construct was subcloned into pDM4 (33). The resulting plasmid pRC302 was transformed into *E. coli* SY327 λ*pir* and SM10 λ*pir* (34), and transferred into CMM2101 by conjugation. Using the same methods, the P_{wza}::*lacZ* construct was introduced into *crp*-deleted RC100, and the resulting strain was named RC344. 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℃ 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℃ 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℃ 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.

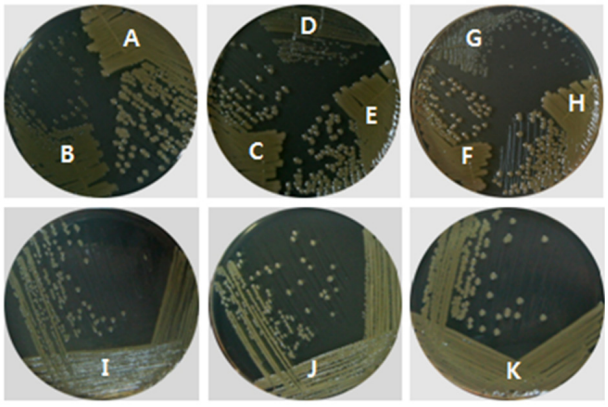
Measurements of bacterial growth and β-galactosidase activity

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℃ 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



A: Wild-type
B: Wza-
C: Wild-type
D: Cya-
E: Cya+
F: Wild-type
G: Crp-
H: Crp+
I: Cya-
J: Cya- (100 μM cAMP)
K: Cya- (500 μM cAMP)

Figure 1. Colony morphologies of *Vibrio vulnificus* strains under different conditions. *V. vulnificus* strains were spread on the surfaces of HI agars or HI agars containing 100 μM cAMP, and cultured for 24 h at 37℃. Wild-type: M06-24/O, Wza-: RC398 containing mutated *wza*, Cya-: RC386 containing mutated *cya*, Cya+: RC390 containing complemented *cya*, Crp-: CMM710 containing mutated *crp*, Crp+: CMM714 containing complemented *crp*.

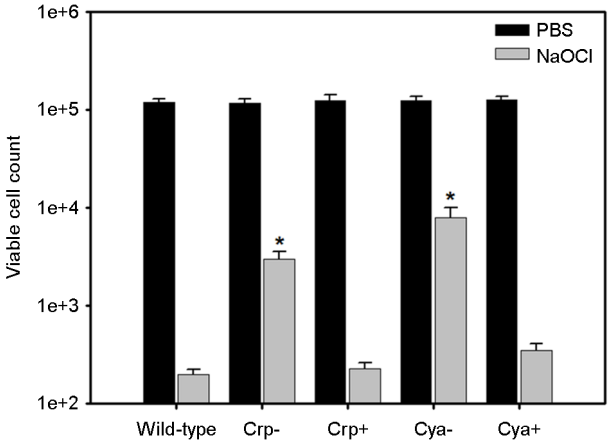


Figure 2. Effect of *cyaA* or *crp* mutation on chlorine resistance. For each *V. vulnificus* strain, approximately 10^5 mid-exponential-phase cells were exposed to either phosphate-buffered saline (PBS, pH 7.2) or 1.5 μg/ml NaOCl for 5 min, which was followed by dilution and plating. The experiments were conducted at least three times, and viable cell count was expressed as the log mean (large bar) and standard deviation (error bar) of triplicate measurements (*: $p < 0.05$ in two-way ANOVA). Wild-type: M06-24/O, Crp-: CMM710 containing mutated *crp*, Crp+: CMM714 containing complemented *crp*, Cya-: RC386 containing mutated *cya*, Cya+: RC390 containing complemented *cya*.

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* stains, 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.

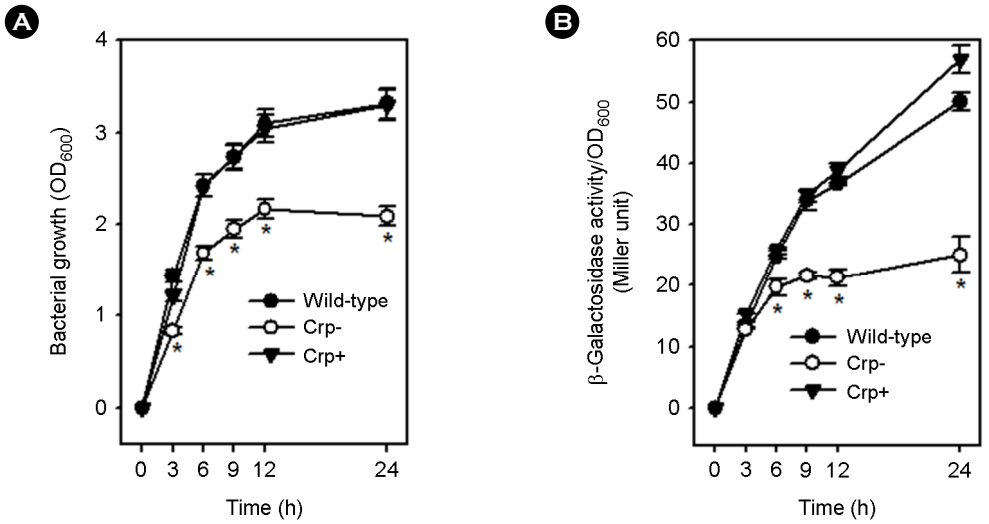


Figure 3. Effect of Crp on the expression of group 1 CPS operon. The three strains containing the P_{wza}::*lacZ* fusion construct, that is, RC342 containing wild-type *crp* (Wild-type), RC344 containing mutated *crp* (Crp-) and RC360 containing complemented *crp* (Crp-), were cultured in HI broths for 24 h at 37℃. Bacterial growths were determined by measuring optical densities of culture aliquots at 600 nm (OD_{600}), and *wza* transcription was quantified by measuring β-galactosidase activity on a per cell basis (Miller units) in culture aliquots. Bacterial growth levels and β-galactosidase activities are the means and standard deviations (error bars) of triplicate measurements (*: $p < 0.05$ in Student's *t*-test).

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_{wza}::*lacZ* transcription reporter strains in HI broths, and by comparing their growth and *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 *in trans* complemented *crp* ($p < 0.05$, Student's *t*-test) (Fig. 3A). The *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 *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 *Vibrio 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

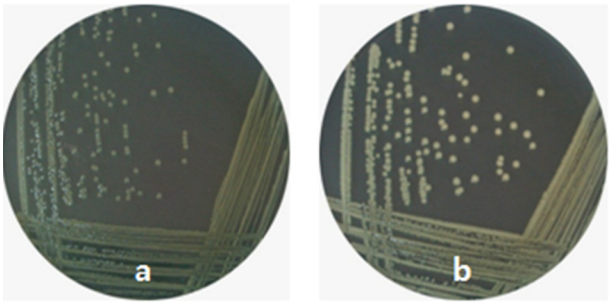


Figure 4. Effect of glucose on the colony opacity of *Vibrio vulnificus* in the absence of Crp. CMM714 containing mutated *crp* was spread on the surface of TES-HI agars containing 0.0 (a) or 0.25% (b) glucose and cultured at 37℃ overnight.

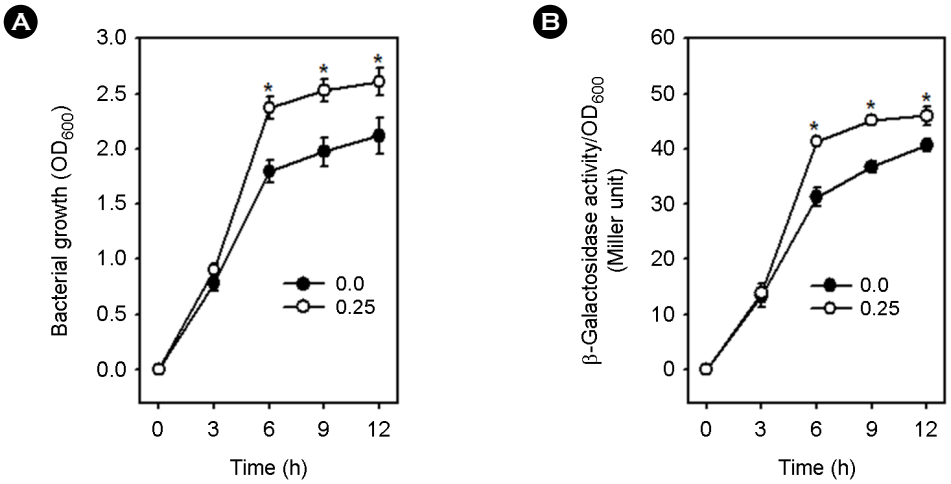


Figure 5. Effect of glucose on *Vibrio vulnificus* growth (A) and the expression of group 1 CPS operon (B) in the absence of Crp. RC344 containing mutated *crp* and the P_{wza}::*lacZ* fusion construct was cultured in TES-HI broths containing 0.0 or 0.25% glucose for 24 h at 37℃. Growth and *wza* transcription were determined and expressed as described in the legend of Fig. 2 (*: $p < 0.05$ in Student's *t*-test).

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_{wza}::*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_6)-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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