J. Microbiol. Biotechnol. (2016), 26(3), 627–636
http://dx.doi.org/10.4014/jmb.1512.12039

jmb

Cholera Toxin Production Induced upon Anaerobic Respiration is Suppressed by Glucose Fermentation in *Vibrio cholerae*

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Received: December 15, 2015
Revised: December 28, 2015
Accepted: December 30, 2015

First published online
December 31, 2015

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pISSN 1017-7825, eISSN 1738-8872

The causative agent of pandemic cholera, *Vibrio cholerae*, infects the anaerobic environment of the human intestine. Production of cholera toxin (CT), a major virulence factor of *V. cholerae*, is highly induced during anaerobic respiration with trimethylamine N-oxide (TMAO) as an alternative electron acceptor. However, the molecular mechanism of TMAO-stimulated CT production is not fully understood. Herein, we reveal that CT production during anaerobic TMAO respiration is affected by glucose fermentation. When the seventh pandemic *V. cholerae* O1 strain N16961 was grown with TMAO and additional glucose, CT production was markedly reduced. Furthermore, an N16961 Δ*crp* mutant, devoid of cyclic AMP receptor protein (CRP), was defective in CT production during growth by anaerobic TMAO respiration, further suggesting a role of glucose metabolism in regulating TMAO-mediated CT production. TMAO reductase activity was noticeably decreased when grown together with glucose or by mutation of the *crp* gene. A CRP binding region was identified in the promoter region of the *torD* gene, which encodes a structural subunit of the TMAO reductase. Gel shift assays further confirmed the binding of purified CRP to the *torD* promoter sequence. Together, our results suggest that the bacterial ability to respire using TMAO is controlled by CRP, whose activity is dependent on glucose availability. Our results reveal a novel mechanism for the regulation of major virulence factor production by *V. cholerae* under anaerobic growth conditions.

Keywords: *Vibrio cholerae*, cholera toxin, anaerobic respiration, glucose metabolism

Introduction

For enteric pathogenic bacteria, physiological changes are essential to maintain viability and pathogenicity by adapting to various host environments, including nutrient fluctuation and oxygen limitation [28, 36]. *Vibrio cholerae*, the causative agent of the pandemic disease cholera, is a facultative anaerobic pathogenic bacterium [7, 16]. Although the pathogenesis caused by this deadly bacterium takes place in the intestine, which is commonly thought to be an anaerobic environment, the mechanism of virulence gene expression under anaerobic conditions is not fully understood.

The ability of *V. cholerae* to colonize and cause disease in the host requires the production of virulence factors during infection, including cholera toxin (CT) and toxin coregulated pilus (TCP). CT provokes watery diarrhea by the generation of ion transport imbalance across the intestinal epithelium, and the type IV TCP contributes to intestinal colonization [19, 41]. Expression of both genes is regulated by a variety of factors that are able to respond to environmental signals. An AraC-type master transcription factor, ToxT, which is regulated by the membrane-associated proteins ToxR and TcpP/TcpH, directly activates transcription of the *ctxAB* genes (encoding CT) and the genes for TCP biogenensis [4, 5, 9, 24, 25, 38]. In addition, the activation of *tcpP/H* (genes encoding TcpP and TcpH, respectively) expression requires two transcriptional activators, AphA and AphB [3, 17]. However, regulation of the ToxT regulon that mediates CT production was defined using strains grown in AKI media, an in vitro culture condition known to be permissive for CT production [15]. Several studies demonstrated that AphB,

the key transcriptional activator of the *tcpP* gene, activates virulence gene expression through anaerobiosis-induced dimerization and activity [18, 22]. In addition, a previous study showed that the production of three colonization factors (TcpC, TcpQ, and TcpS) and accessory colonization factor A (AcfA) is induced under anaerobiosis [23]. Together, these results suggest that oxygen limitation may be the key to regulating pathogenic processes of *V. cholerae* during host intestinal infection.

Recently, we reported that CT production is substantially enhanced by anaerobic respiration using trimethylamine *N*-oxide (TMAO) as an alternative electron acceptor, and it is mediated by the accumulation of guanosine pentatetraphosphate (termed (p)ppGpp), also known as a bacterial stress alarmone [21, 34]. Meanwhile, our recent report demonstrated that (p)ppGpp confers a growth and survival advantage through acetoin fermentation, a known *V. cholerae* strategy to avoid fatal acidification of the growth environment in glucose-enriched environments [33, 43]. Up to 70–80% of cholera patients recover by treatment with the continuous administration of oral rehydration solution (ORS). Because a large amount of glucose is present in ORS, it is speculated that continuous administration of ORS may create glucose-enriched microenvironments in the human intestine [6, 10, 35, 37]. Subsequently, the growth and virulence of *V. cholerae* may be influenced by glucose metabolism under anaerobic conditions. Therefore, we predicted that the (p)ppGpp-mediated CT production and growth advantage are diminished by glucose in the human intestine. Until now, the relationship between glucose metabolism and virulence regulation under anaerobic conditions has not yet been explored.

In this study, we revealed that the utilization of TMAO in anaerobic respiration, which stimulates CT production, is repressed by glucose fermentation. This work is an extension of our previous study and describes a regulation mechanism for the production of CT under anaerobic conditions. This report provides evidence that the production of CT, an important virulence factor, is critically controlled by anaerobiosis-induced glucose metabolism.

Materials and Methods

Bacterial Strains and Growth Conditions

All bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in lysogeny broth medium (LB; 1% tryptone, 0.5% yeast extract, and 1% NaCl per liter), and antibiotics were used in the following concentrations; ampicillin, 100 μg/ml; streptomycin, 200 μg/ml; and kanamycin, 50 μg/ml. To support anaerobic growth, 50 mM TMAO (Sigma Aldrich Co., St. Louis, MO, USA) was added to the medium (termed LBT). The effect of glucose was assessed using cells grown in LB or LBT containing 1% glucose (termed LBG and LBTG, respectively). An overnight culture of bacterial cells was subcultured at a 1/100 dilution in fresh medium and grown at 37°C in an anaerobic chamber. Bacterial growth was monitored spectrophotometrically by measuring the optimal density at 600 nm, and the pH of the culture media was monitored using a pH meter.

Transposon Mutagenesis and Dot Blot Assay

Construction of a transposon insertion mutant library was performed following previously described procedures [21]. N16961-derived mutants were grown in 96-well plates anaerobically in LBT. After overnight growth, culture supernatants from each well

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Ref. or source
V. cholerae strains		
N16961	Wild type, O1 serogroup, El Tor biotype	Lab. collection
C6706	Wild type, O1 serogroup, El Tor biotype	Lab. collection
O395	Wild type, O1 serogroup, classical biotype	Lab. collection
crp::Tn	N16961, Tn inserted in coding region of *VC2614* gene	This study
Δcrp	N16961, *crp* gene deleted	This study
E. coli strains		
SM10/λpir	Km^r *thi-1 thr leu tonA lacY supE recA*::RP4–2-Tc::Mu *pir*$^+$, for conjugal transfer	Lab. collection
BW20767	Km::Tn*7 leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(ΔMlu1)::*pir*$^+$*thi*	Lab. collection
BL21b	*sup*E44 D*lac*U169 (f80*lacZ* DM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Lab. collection
Plasmids		
pCVD442	*sacB* suicide vector from plasmid pUM24	[21]
pBTK30	Transposon insertion vector, Gm^r	[26]

Table 2. Primers used in this study.

Gene name	Direction	Primer sequence (5′-3′)[a]	Restriction sites
Cloning			
crp (His-tag)	Forward	ACCTTCATATGGTTCTAGGTAAACCTCAAACC	NdeI
	Reverse	TAGAGCTCGAGGCGAGTGCCGTAAACCACGATG	ZhoI
crp left (in-frame deletion)	Forward	CTCTAGAGCTCGCGTGTTCCTACGGTCGATA	SacI
crp left (in-frame deletion)	Reverse	TGGAAGGATCCTGGATCGGTTTGAGGTTTACC	BamHI
crp right (in-frame deletion)	Forward	CTCTAGGATCCGTGGTTTACGGCACTCGCTA	BamHI
crp right (in-frame deletion)	Reverse	TGGAAGAGCTCCAAGCATTAGCCAGTGAGGC	SacI
Arbitrary PCR			
BTK30-Tnp1, 1 round	Forward	CACCGCTGCGTTCGGTCAAG	
RP-1, 1 round	Reverse	CTTACCAGGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	
RP-2, 1 round	Reverse	CTTACCAGGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC	
BTK30-Tnp2, 2 rounds	Forward	CGAACCGAACAGGCCTTATGTTCAATTC	
RP-3, 2 rounds	Reverse	CTTACCAGGCCACGCGTCGACTAGTAC	
BTK30-Tnp3	Sequencing	TGGTGCTGACCCCGGATGAAG	

[a]Restriction enzyme recognition sequences are underlined.

were spotted on a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA) using a 96-well pin replicator. Membranes were then blocked and probed with a rabbit polyclonal antibody against CT subunit B (Abcam Inc., Cambridge, UK) following a standard western blot protocol. To determine the location of the transposon insertion site, arbitrary PCR was performed with the primers listed in Table 2.

Construction of Mutant Strain

The *crp* in-frame deletion mutants were created by allele replacement as previously described [21]. The 500 base pair flanking sequences located at both ends to introduce mutation were amplified by PCR with the primers listed in Table 2.

CT ELISA Assays

CT production was measured using *V. cholerae* culture supernatants by GM_1-enzyme-linked immunosorbent assays (GM_1-ELISA) as described previously [39]. Purified CT subunit B (List Biological Laboratories Inc., Campbell, CA, USA) was used to provide a standard curve and phosphate-buffered saline (PBS) was used as a negative control. Rabbit polyclonal anti-CT subunit B (Abcam Inc.) and anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Santa-Cruz Biotechnology Inc., Dallas, TX, USA) were used for detection.

Microarray Analysis

Differential gene expression analysis was performed using Roche NimbleGen Vcho expression arrays (4 × 72K), consisting of 72,000 probes representing 3,835 ORFs from the *V. cholerae* N16961 strain. The *V. cholerae* N16961 strain was anaerobically grown in LB, LBT, or LBTG for 6 h. Total bacterial RNA was isolated from each of the three independent cultures and RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON, Canada) following the manufacturer's instructions. Extracted RNA was further purified using an RNeasy kit (Qiagen). Purified RNA samples were then pooled together and submitted to GenoCheck Inc. (Ansan, South Korea) for subsequent experimentation. Slides were scanned using the Axon 4000B microarray scanner system, and images were processed using NimbleScan 2.4 software (Roche Technologies). For differentially expressed gene selection, GeneSpring (ver. 11.5.1) was used.

Purification of CRP

For recombinant protein production, the CRP-encoding gene from N16961 was PCR-amplified and positionally cloned into pET21b. The primer set used for cloning is listed in Table 2. The resulting plasmid was then transformed into *E. coli* BL21(DE3). Then 1 mM IPTG was used to induce overexpression, and recombinant hexahistidine-tagged (6His) proteins were purified using Ni-NTA agarose (Qiagen). Protein purity was >95%, as determined densitometrically after separation by SDS-PAGE and staining with Coomassie blue. The Bradford method was used to determine the concentration of 6His-CRP with comparison against a standard curve generated with bovine serum albumin. Purified proteins were stored at –20°C to prevent degradation.

Electrophoretic Mobility Shift Assay

Complimentary oligonucleotides (40 bp) that contain the region of putative CRP binding sequences were synthesized with a 5′-biotinylated modification (Macrogen Inc., Seoul, Korea). Mixed complementary oligonucleotides (1:1 molar ratio) were incubated at 95°C for 5 min followed by a gradual reduction in heat until the

oligonucleotides reached room temperature. Purified 6His-CRP (300 nM) was incubated with 1 nM biotin-labeled DNA and 1 μM cAMP in binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 5% (v/v) glycerol, and 50 ng/μl poly(dI·dC)) for 15 min at 20°C. For the competitor DNA, a 10-, 50-, or 100-fold molar excess of unlabeled target DNA was added to each reaction mixture, respectively, with the extract before the labeled DNA target was added. The mixtures were size fractionated on a non-denaturing 5% polyacrylamide gel, followed by drying and transfer onto nitrocellulose membranes. Signals were detected by streptavidin-horseradish peroxidase chemiluminescence for biotin-labeled probes according to the manufacturer's instructions (Pierce). The images were visualized using the ChemiDoc XRS system with image software (Bio-Rad).

TMAO Reductase Activity Assay

V. cholerae strain N16961 and the *crp* mutant were grown anaerobically in LB, LBG, LBT, or LBTG for 6 h. To analyze TMAO reductase activity in different cellular fractions, the periplasmic and cytoplasmic fractions were separated by polymyxin B treatment. Cell pellets were resuspended with PBS containing 2,000 units of polymyxin B and incubated for 15 min at 4°C. After incubation, the reaction mixtures were centrifuged at 12,000 rpm for 10 min and the supernatants were collected for periplasmic fractions. The cell pellets were then resuspended in PBS and sonicated to produce cytoplasmic fractions. Equal amounts of proteins present in each fraction were resolved on a 9% non-denaturing polyacrylamide gel, and a native gel-based enzyme assay was performed as previously described [44].

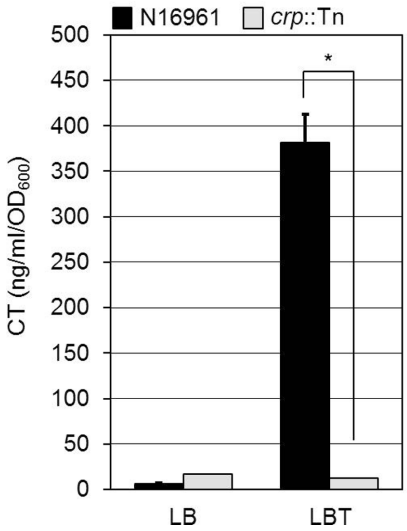

Fig. 1. CT production was decreased in the *crp*::Tn mutant during anaerobic TMAO respiration.

Wild-type N16961 and the *crp*::Tn mutant were inoculated in separate LB and LBT (LB containing 50 mM TMAO) cultures and grown statically in an anaerobic chamber for 16 h. Culture supernatants were harvested and the CT level was quantified by ELISA. *$p < 0.05$ vs. CT levels produced from wild-type N16961. Three independent experiments were performed and the mean ± SD represented in the bar graph.

Confocal Microscopy

Differential interference contrast (DIC) and green fluorescent images were acquired using a confocal laser scanning microscope (FV-1000; Olympus Optical Co. Ltd., Japan) and its operating software, FV10-ASW (ver. 02.01). Detailed procedures are described elsewhere [20]. For reactive oxygen species (ROS) detection, bacterial cells were grown anaerobically for 4 h with 50 mM TMAO or 1% glucose. Aliquots of each culture were removed and stained with 10 μM DCF-DA (2′,7′-dichlorofluorescein diacetate; Sigma) for 30 min. To capture the green fluorescence, samples were scanned at 488 nm and emission was detected through a 520 nm band filter. The DIC and green fluorescence images were collected simultaneously.

Statistical Analysis

Data are expressed as the mean ± SD. An unpaired Student's *t* test was used to analyze the data. A *p*-value of <0.05 was considered statistically significant. All experiments were repeated for reproducibility.

Results

Identification of a *V. cholerae* Mutant That Lost the Ability to Produce CT During Anaerobic TMAO Respiration

Our previous studies showed that *V. cholerae* O1 serotype strains produced a high level of CT during anaerobic TMAO respiration, in a manner dependent on the stringent response [21, 34]. These results uncovered a novel growth condition for *V. cholerae* virulence and provided an insight into the basis of elevated CT production. To better understand the mechanistic details, we constructed a transposon (Tn) insertion mutant library and isolated a mutant that became defective in CT production under the same growth condition that permitted robust CT production in the N16961 parental strain. Over 1,200 mutants were screened by a dot-blot analysis using an antibody against CT subunit B. One mutant that harbored a transposon insertion in the *VC2614* gene was validated (Fig. 1). The *VC2614* gene encodes cAMP receptor protein (CRP, also known as catabolite activator protein), which regulates genes involved in carbon source metabolism [2, 30]. The *crp*::Tn mutant was clearly defective in CT production when grown anaerobically in the presence of 50 mM TMAO (Fig. 1). N16961 cells produced a significant level of CT under the same growth condition. CT production was not observed in N16961 cells grown in plain LB (Fig. 1). This result further confirms that CT production occurs during anaerobic TMAO respiration and suggests that CRP

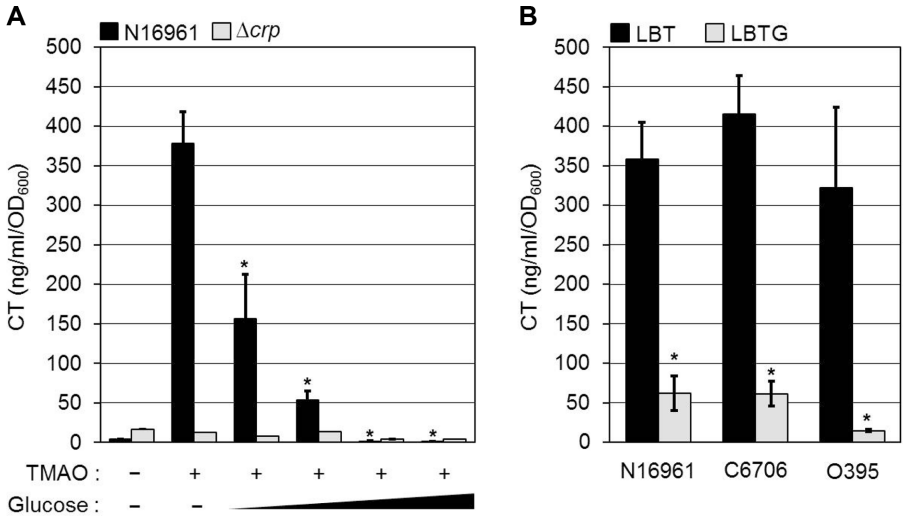

Fig. 2. Glucose represses TMAO-stimulated cholera toxin production.
(**A**) Effect of the presence of glucose on CT production. N16961 cells were grown anaerobically in LBT for 16 h with 2-fold serially diluted 2% glucose and culture supernatants were assayed for CT ELISA. *$p < 0.05$ vs. CT produced in N16961. (**B**) The levels of CT produced in various strains grown anaerobically in LBT or LBTG (LBT containing 1% glucose) for 16 h. *$p < 0.05$ vs. CT levels produced under LBT growth conditions.

is closely linked to the mechanism of TMAO-stimulated CT production.

CT Production Is Repressed by Glucose During TMAO-Induced Anaerobic Respiration

As a transcriptional activator, CRP functions in conjunction with cAMP. When bound to cAMP, CRP-mediated transcriptional regulation occurs more specifically [11], and in *E. coli*, intracellular cAMP levels are inversely correlated with medium glucose concentration [32]. These notions led us to postulate that the phenotypes observed in the *V. cholerae crp*::Tn mutant with respect to CT production may be similar to those found in the wild-type strain under glucose-rich conditions. To address this issue, we grew bacterial strains anaerobically in the presence of both 50 mM TMAO and varying concentrations of glucose. As shown in Fig. 2A, CT production gradually decreased when the levels of added glucose increased (black bars). In the presence of 1% glucose, only a negligible amount of CT was produced in N16961. Consistently, CT production was not detectable in the Δ*crp* mutant (gray bars). CT production induced upon anaerobic TMAO respiration was also detected in C6706 (another El Tor biotype) and O395 (a classical O1 serotype strain) (Fig. 2B). When grown with 1% glucose, CT production was markedly decreased in these two strains (Fig. 2B), suggesting that the glucose-mediated suppression of CT production is consistent in related *V. cholerae* strains.

Expression of Genes Involved in TMAO Respiration Decreased in the Presence of Glucose in a CRP-Dependent Manner During Anaerobic Respiration

We performed a microarray analysis to elucidate the effect of added glucose on genome-wide expression profiles. When grown with glucose, the expression of genes involved in the production of functional TMAO reductase was substantially decreased (Table 3). Expression of these genes was highly induced in cells grown anaerobically in LBT. For example, the *torA* gene encoding a structural component of the TMAO reductase was actively transcribed when grown with TMAO. The level of its transcription was increased by more than 20-fold in N16961 cells grown anaerobically by TMAO respiration; however, when grown in LBTG medium, transcription decreased back to the level found in cells grown in plain LB (Table 3). These results suggest that the presence of glucose suppresses the ability of *V. cholerae* to grow by anaerobic TMAO respiration. To further address this notion, we measured TMAO reductase activity in N16961 and its Δ*crp* mutant. Consistent with results in Table 3, TMAO reductase activity was substantially decreased in N16961 cells grown in LBTG compared with LBT-grown cells (Fig. 3). When grown in LBT, a condition that activates anaerobic growth by TMAO respiration, TMAO reductase activity was not induced in the Δ*crp* mutant (Fig. 3). These findings further demonstrate that a lack of CT production in the mutant is tightly associated

Table 3. Microarray analysis of genes affected by glucose during TMAO respiration.

Gene No.	LB signal value	LBT signal value	LBTG signal value	Product name	Function
VC1692	351.4164	7,153.128	413.4143	TorA	Trimethylamine *N*-oxide reductase
VC1693	433.8665	9,246.063	517.9152	TorC	Cytochrome *c*-type protein
VC1719	341.936	5,51.0156	312.8968	TorR	DNA-binding transcriptional regulator
VC1720	266.2405	4,926.492	304.7419	TorD	Chaperone protein

Microarray analysis reveals that the expression of genes involved in TMAO utilization is decreased by glucose during anaerobic respiration.

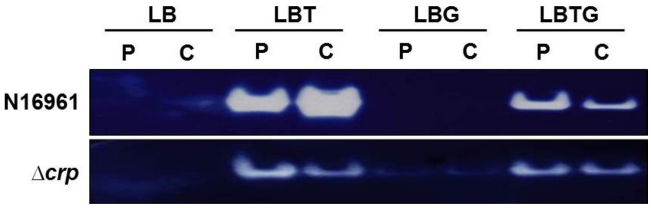

Fig. 3. TMAO reductase activity decreased by glucose or by mutation of the *crp* gene.

Bacterial strains were grown anaerobically in LB, LBT, LBG, or LBTG for 6 h. Proteins present in the cytoplasmic (C) and periplasmic (P) fractions were separated by native gel electrophoresis and stained for TMAO reductase activity as described in the Materials and Methods.

with its defect to activate TMAO reductase.

We then sought to understand how bacterial strains respond to diverse growth conditions. N16961 appeared to grow better anaerobically in LBG vs. LB (Fig. 4A). However, a sharp decrease in medium pH was observed in LBG (Fig. 4B) and acidification-induced loss of viability was observed (data not shown). When grown in LBT, the medium pH increased owing to the accumulation of trimethylamine (TMA), the product of TMAO reductase (Fig. 4B). Of note is that N16961 grew most actively in LBTG (Fig. 4A). Under this condition, medium acidification was not as severe as observed in LBG (Fig. 4B). Interestingly, anaerobic growth of the Δ*crp* mutant in LB was not as active as its parental strain, N16961 (Fig. 4A). Furthermore, anaerobic growth of the mutant in LBT was severely delayed compared with N16961 (Fig. 4A). This is likely ascribed to reduced TMAO reductase activity incurred by mutation of the *crp* gene (Fig. 3).

cAMP-CRP Complex Binds to the Shared Promoter Between the Divergently Transcribed *torR* and *torD* Genes

Our results suggest that CRP positively regulates the expression of genes involved in TMAO reductase. It is of note that the *torR* and *torD* gene promoter region contains a potential CRP recognition sequence (Fig. 5A). To determine whether CRP binds to this genetic element, we performed an electromobility shift assay. As shown in Fig. 5B, purified CRP, in conjunction with cAMP, bound directly to the

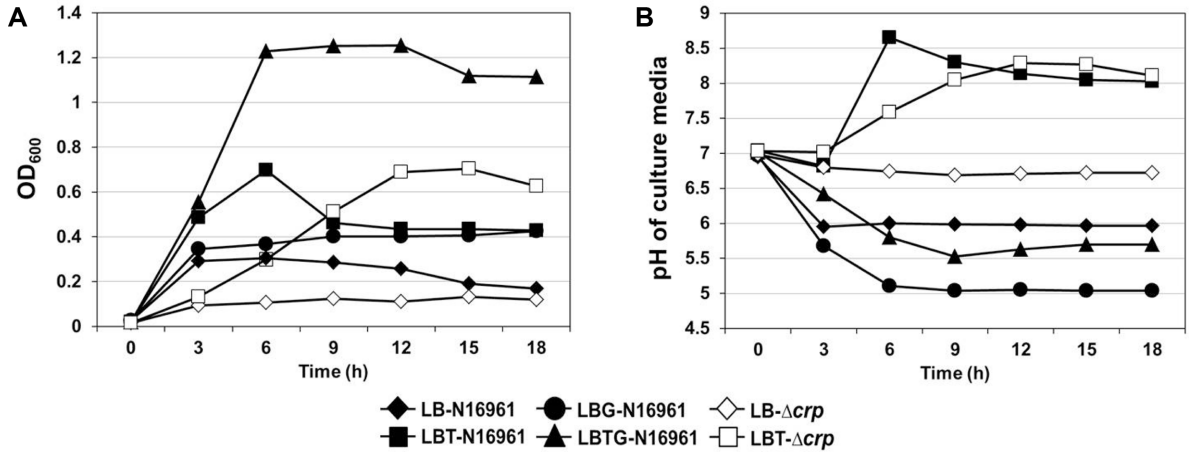

Fig. 4. Bacterial growth and pH changes of the culture media.

Various growth-associated parameters of the N16961 (black) and *crp* deletion mutant (white) strains were measured every 3 h during growth in LB (diamonds), LBT (squares), LBG (circle), or LBTG (triangle). (**A**) Aliquots of each culture were withdrawn at the designated times and the OD_{600} values were plotted for growth curves. (**B**) The medium pH was measured in aliquots of culture supernatants and was plotted with respect to time.

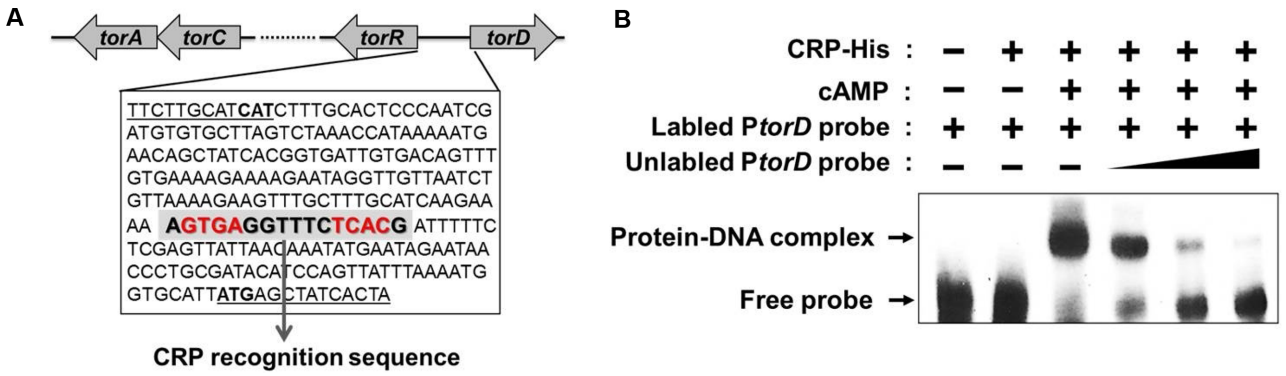

Fig. 5. Gel mobility shift assay of the *torD* promoter region using 6His-CRP.
The cAMP-CRP complex interacts with the *torD* promoter. (**A**) The sequence of the CRP binding sites at the *torD* promoter region. (**B**) A biotinylated DNA fragment of the *torD* promoter was incubated with purified 6His-CRP as described in the Materials and Methods. Unlabeled competitor DNA probes were added to the binding reaction at a 10-, 50-, or 100-fold excess relative to the biotinylated *torD* DNA probe. Equal aliquots of the binding reaction mixture were then electrophoresed on non-denaturing 5% acrylamide gels and the position of the *torD* probe was visualized by streptavidin-horseradish peroxidase chemiluminescence.

promoter. The protein without extraneously added cAMP failed to bind to the promoter sequence (Fig. 5B, second lane). Furthermore, this binding was specific since non-labeled promoter DNA was able to compete for CRP binding. Together, these data strongly indicate that CRP positively regulates transcription of *torR* and *torD*, two genes important for the production of functional TMAO reductase.

Production of Intracellular ROS, Generated During Anaerobic TMAO Respiration, Is Diminished when Grown Together with Glucose or by Mutation of the *crp* Gene

Our previous results suggested that ROS are produced during anaerobic TMAO respiration and CT production is likely an outcome of bacterial responses to ROS [21]. Since our current study revealed a suppressive role of glucose in CT production, we examined whether ROS-specific fluorescent signals could be detected in bacterial cells grown with added glucose. Fig. 6 clearly demonstrates that DCF-DA signals were detected only in N16961 grown anaerobically in LBT. ROS-specific signals were not detectable in N16961 grown with TMAO and glucose (Fig. 6). In addition, the Δ*crp* mutant cells, incapable of producing CT during anaerobic TMAO respiration, did not produce ROS-specific signals when grown in LBT (Fig. 6). Together, these results further corroborate that glucose

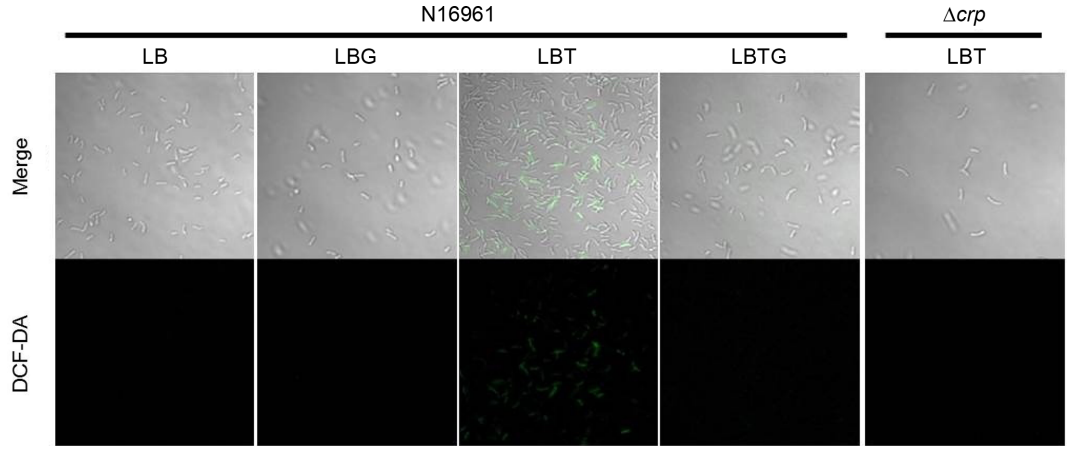

Fig. 6. The level of intracellular ROS was decreased by glucose or by mutation of the *crp* gene during anaerobic TMAO respiration.
Confocal microscope images. Bacterial cells grown anaerobically in LB, LBG, LBT, or LBTG. Upper images in each panel represent merged DIC and green fluorescent images, whereas lower images represent only green fluorescent images. Bacterial cells grown in each medium for 4 h were stained with 10 μM DCF-DA for 50 min and processed for confocal microscopic analysis. Images were acquired at 1,000× magnification.

metabolism created an environment that suppresses TMAO respiration, ROS generation, and thus CT production.

Discussion

V. cholerae can grow by carbohydrate fermentation [31]. When grown with glucose, the *V. cholerae* O1 El Tor biotype strain can activate the 2,3-butanediol fermentation pathway to fully catabolize available glucose. Since 2,3-butanediol is a neutral fermentation end-product, uninterrupted production of 2,3-butanediol can prevent medium acidification and therefore maintain cell viability [43]. Under anaerobic conditions, *V. cholerae* can also support growth by respiration using alternative electron acceptors, such as TMAO and fumarate [21]. We previously demonstrated that CT production was highly induced in *V. cholerae* strains grown by anaerobic TMAO respiration [21]. This finding uncovered a novel growth condition that leads to robust CT production and supports the idea that bacterial virulence is tightly associated with energy metabolism [27]. However, little attention has been paid to (i) how *V. cholerae* manages its anaerobic growth when both growth modes (*i.e.*, fermentation and respiration) are available, and (ii) how its virulence is affected under each anaerobic growth condition.

In this work, we elucidated the effect of glucose metabolism on CT production, triggered by anaerobic TMAO respiration. Under anaerobic conditions, N16961 lost viability owing to medium acidification as a result of glucose fermentation, suggesting that the 2,3-butanediol fermentation pathway may not be operational anaerobically (Fig. 4B). When grown with TMAO, a compound that stimulates anaerobic respiration, N16961 also lost viability, likely due to the overproduction of ROS, while CT production was greatly induced [34]. These two findings suggest that *V. cholerae*, although classified as a facultative anaerobe [23], cannot optimally propagate under anaerobic conditions. In addition, anaerobic growth in plain LB was not robust (Fig. 4A). We postulate that such defects in anaerobic growth may account for the ability of *V. cholerae* to only cause acute infection in the anaerobic human intestine, with a short incubation period of normally less than 7 days [1, 8].

Based on our results shown in Fig. 3 and Table 3, TMAO reductase activity was substantially decreased in N16961 grown with both TMAO and glucose. This result suggests that stimulation of fermentative growth by glucose suppresses respiratory growth using TMAO. Ironically, however, bacterial growth in the presence of both glucose and TMAO was most active (Fig. 4A), due to a pH neutralization effect by the production of TMA, which serves as a base. TMA production mitigated the medium acidification elicited by the accumulation of organic acids. These findings suggest that (i) *V. cholerae* responds quite sensitively to changes in medium pH, and (ii) the two distinct growth modes (*i.e.*, fermentation and respiration) are not cooperative in supporting anaerobic growth of *V. cholerae*. Catabolite repression induced by glucose often occurs to suppress metabolism of other carbon sources [40]. In *E. coli*, the production of glycerol-3-phosphate dehydrogenase, an enzyme involved in anaerobic electron transfer, was reported to require cAMP, suggesting that cAMP plays a role in regulating anaerobic metabolism [42]. Our results show that intracellular levels of cAMP can positively control the production of TMAO reductase, a major anaerobic respiratory enzyme. To our best knowledge, this is the first demonstration that catabolite repression can occur between fermentative and respiratory growth in *V. cholerae*.

The stringent response, a bacterial adaptation response to nutrient starvation, was shown to be activated during anaerobic TMAO respiration [34]. This suggests that CT production in *V. cholerae* is stimulated when cells encounter adverse growth conditions. When glucose was added, anaerobic growth of N16961 was significantly increased (Fig. 4A), and at the same time, CT production was almost completely abrogated. These results further suggest that CT production inversely correlates with the degree of bacterial growth. It is of note that oral rehydration solution, a primary therapy for cholera, contains a large amount of glucose (2% w/v) [29]. ORS treatment results in considerable water absorption from the intestinal lumen [13, 14] and a decrease in net stool output is observed in ORS-treated cholera patients [12]. Since the addition of glucose suppressed CT production under a growth condition that otherwise permitted CT production, glucose in ORS may serve as an agent that inhibits CT production in vivo.

In conclusion, we identified a novel effect of glucose metabolism on *V. cholerae* anaerobic growth and virulence. Glucose metabolism exerts a wide range of functions in *V. cholerae*, as it influences bacterial viability depending on oxygen availability and virulence. Our results (summarized in Fig. 7) put a renewed emphasis on understanding *V. cholerae* virulence in association with metabolism. CRP, as a major transcriptional regulator, controls the expression of many genes, including those involved in carbohydrate catabolism. Here, two more genes encoding TorR and TorD are listed as part of the CRP regulon. Future work describing the intricate regulatory pathways between respiratory and fermentative growth and the impact of growth condition on virulence factor production is definitely warranted to

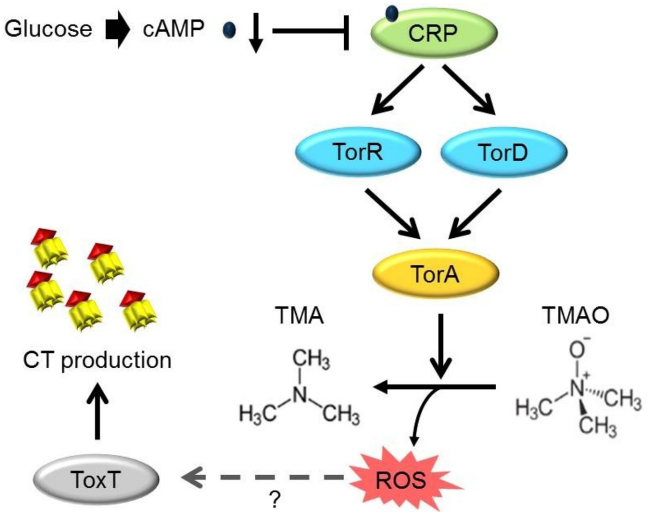

Fig. 7. Potential mechanism for the glucose effect under anaerobic TMAO-respiration-induced CT production.
Cholera toxin production induced upon anaerobic TMAO respiration is suppressed by glucose fermentation. *V. cholerae* can grow via anaerobic respiration using TMAO under oxygen-limited conditions. Production of CT is highly promoted during TMAO-specific anaerobic respiration [21, 34]. The ability to respire using TMAO is controlled by the cAMP-CRP complex, which regulates the activity of TorA via positive regulation of *torD* and *torR* expression. However, when *V. cholerae* encounters a glucose-rich environment under the same conditions, intracellular cAMP levels become low and the activity of TorA is subsequently decreased. Overall, the *V. cholerae* growth mode is mainly converted to glucose by fermentation, and a decrease in the ability to use TMAO leads to a reduction in CT production.

better propose efficient strategies to combat *V. cholerae* infection.

Acknowledgments

This work was supported by grants from the National Research Foundation (NRF) of Korea, funded by the Korean Government (MSIP) (2014R1A2A2A01002861, 2014R1A4A1008625, and 2014R1A1A2056139). This work was also supported by Yonsei University College of Medicine, Dongwha Holdings Faculty Research Grant (6-2014-0057). The authors have declared no conflicts of interest.

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