理學碩士學位論文

Host-derived glucose activates quorum sensing signals and the virulence of *Vibrio vulnificus* during their infection

체내 감염 상황에서 비브리오 패혈증균의 혈당 인식에 따른 병원성 조절

2022년 2월

서울大學校 大學院

生命科學部

李在勳

Host-derived glucose activates quorum sensing signals and the virulence of *Vibrio vulnificus* during their infection

by

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Under the supervision of

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A Thesis for the Degree of **Master of Philosophy**

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School of Biological Sciences

Seoul National University

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Abstract

Host-derived glucose activates quorum sensing signals and the virulence of *Vibrio vulnificus* during their infection

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Bacteria recognize and respond to a variety of extracellular environments. Quorum sensing is a cell-to-cell communication process that allows bacteria to regulate genes in response to changes in surrounding bacterial cell density and species composition. In *Vibrio cholerae*, VqmA-VqmR quorum sensing pathway regulates pathogenesis and senses host-derived signals. Here, I identify the function of VqmA-VqmR pathway in *Vibrio vulnificus*. Transcription factor VqmA activates expression of *vqmR* and affects pathogenicity in the mouse infection model. The expression level of *vqmR* is increased in the presence of glucose because dephosphorylated HPr interacts with VqmA and increases the transcriptional activity of VqmA. The Interaction between HPr and VqmA is highly species-specific. I propose that *Vibrio vulnificus* recognizes the infection niche through the interaction

between VqmA and HPr and regulates pathogenicity.

Key words:

Quorum sensing, PEP:carbohydrate phosphotransferase system (PTS), VqmA, small regulatory RNA, VqmR, HPr, glucose

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Abbreviations

QS, quorum sensing

AI, autoinducer

V. vulnificus*, *Vibrio vulnificus

V. cholerae*, *Vibrio cholerae

DPO, ,5-dimethylpyrazin-2-ol

Tdh, threonine dehydrogenase

PTS, Phosphoenolpyruvate: carbohydrate phosphotransferase system

EI, enzyme I of the PTS

HPr, histidine-containing phosphocarrier protein

$EIIA^{Glc}$, glucose specific-enzyme IIA subunit of PTS

Glc, glucose

sRNA, small RNA

Cys, cysteine

DTT, Dithiothreitol

Mtl, mannitol

Gly, glycerol

2-DG, 2-deoxyglucose

EMSA, electrophoresis mobility shift assay

Chapter I. Literature Review

1. Quorum sensing (QS) system

1.1 Overview of quorum sensing system in gram-negative bacteria

Quorum sensing is the mechanism by which bacteria globally regulate genes in response to the cell density and species composition of the surrounding environment. (Lupp, et al. 2003). Quorum sensing consists of three processes: synthesis, secretion, and group-wide detection of extracellular signaling molecule, which are called autoinducers (Pan and Ren 2009). Bacteria detect the number of surrounding cells by sensing the concentration of autoinducers, thereby changing gene regulation. Mechanisms regulated by quorum sensing include bioluminescence, the secretion of virulence factors, the production of public goods and the formation of biofilms, and these mechanisms are very unproductive when performed by a single bacterial cell (Bassler and Losick 2006).

Quorum sensing system of nearly all known Gram-negative bacteria have four common features (Ng and Bassler 2009). First, Autoinducers bind to specific receptors in the inner membrane or cytoplasm. Second, the quorum sensing pathway regulates a wide variety of genes required for each cell density. Third, When the signal is activated by the autoinducer in the quorum sensing pathway, the synthesis of autoinducer is increased. Fourth, gram-negative bacteria usually produce acyl-homoserine lactones as signaling molecules, and they are able to diffuse freely through the bacterial membrane. When signaling molecules bind to receptors in the bacterial membrane or cytoplasm, the quorum sensing signal pathway is modulated. Phosphorylation-dependent signal transduction or the two-component system are used in these processes. Quorum sensing information is often integrated by small RNAs that regulate various target gene expression (Papenfort and Vogel 2010).

1.2 Quorum sensing system in *Vibrio* species

Vibrio is a genus of Gram-negative bacteria, possessing a curved-rod shape, several species of which can cause foodborne infection, usually associated with eating undercooked seafood (Thompson, et al. 2005; Li and Wang 2020). Quorum sensing plays a crucial role in various infection-related biological processes of marine *vibrio* species, including survival, colonization, invasion, and pathogenesis (Papenfort and Bassler 2016). Although each *vibrio* species has various autoinducers, most of them belong to acyl homoserine lactones. Despite the diversity of signaling molecules and receptors, the quorum sensing pathway in *vibrio* species is mostly mediated by LuxU and LuxO. Depending on the binding of signaling molecule, the receptor activity changes, which leads to phosphorylation of LuxU and LuxO. Phosphorylated LuxO together with the alternative sigma factor σ^N activates the expression of genes encoding four homologous regulatory small RNAs, called Qrr1-4. Qrr small RNAs regulate various target genes such as virulence factors and biofilm formation genes (Ball, et al. 2017; Herzog, et al. 2019).

1.3 Quorum sensing dependent regulation of virulence in *Vibrio* species

Quorum sensing is crucial for virulence in Gram-negative bacteria. Qrr small RNA-mediated regulation of virulence is important in *vibrio* species during their infection (Herzog, et al. 2019). In *Vibrio cholerae*, qrr, which is activated at low cell densities, regulates the transcription factor HapR and AphA (Rutherford, et al. 2011; Xu, et al. 2020). AphA activates the production of the toxin-co-regulated pilus (TCP) and the cholera toxin (CTX) together with another transcription factor AphB. (Kovacikova and Skorupski 2001; Almagro-Moreno, et al. 2015). TCP and CTX play an important role when infecting humans. (Almagro-Moreno, et al. 2015). HapR antagonizes these functions by inhibiting AphA synthesis (Ball, et al. 2017). In *Vibrio vulnificus*,

Qrr small RNA regulates transcription factor SmcR (McDougald, et al. 2001; Elgaml, et al. 2014). SmcR is the main activator of metalloproteinase gene *vvpE* transcription and SmcR can activate the expression of CPS-related genes (Kim, et al. 2013). Recent studies have found that SmcR can negatively regulate the formation of bacterial biofilms which is of great significance for the invasion and pathogenicity of *Vibrio vulnificus* (Miyoshi, et al. 2012; Elgaml, et al. 2014).

1.4 VqmA-VqmR quorum sensing system in *Vibrio* species

Previous studies have revealed that *Vibrio cholerae* has a VqmA-VqmR mediated pathway in addition to the qrr-mediated quorum sensing pathway (Papenfort, et al. 2015). VqmA is a cytoplasmic transcription factor that binds to autoinducer 3,5-dimethylpyrazin-2-ol (DPO) (Papenfort, et al. 2017). DPO is made from threonine and alanine, and its synthesis depends on threonine dehydrogenase (Tdh). The VqmA–DPO complex activates expression of *vqmR*, which encodes a small regulatory RNA. VqmR represses genes required for biofilm formation and virulence (Herzog, et al. 2019). *Vibrio cholerae*'s VqmA detects the lack of oxygen and the presence of bile salts in the small intestine, which allows them to recognize the environment in which they should be pathogenic (Mashruwala and Bassler 2020). *vqmA* and *vqmR* genes are also conserved in other *Vibrio* species, but few studies have been conducted other than *V. cholerae*.

2. Phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS)

2.1 Overview of PTS

The PTS is a multicomponent system that catalyzes the concomitant phosphorylation and translocation of numerous sugar substrates across the cytoplasmic membrane (Kundig, et al. 1964; Postma, et al. 1993). This system consists of two general components, enzyme I (EI) and the histidine-containing phosphocarrier protein HPr, which are common to all PTS sugars, along with many sugar-specific components collectively known as enzyme IIs (EIIs) (Tchieu, et al. 2001; Barabote and Saier 2005).

PTS regulates various cellular functions through phosphorylation-dependent interactions with various proteins (Deutscher, et al. 2014). It has been known that PTS performs several regulatory functions, such as carbon catabolite repression, motility regulation, and biofilm formation, as bacteria recognize whether extracellular sugar is present through the phosphorylation state of PTS components.

2.2 PTS dependent regulation in *Vibrio* species

PTS regulates several mechanisms such as motility and biofilm formation depending on the presence of PTS-sugar. In *Vibrio vulnificus*, dephosphorylated glucose specific EIIA ($EIIA^{Glc}$) is known to interact with a flagella assembly protein (FapA) (Park, Park, et al. 2016; Park, et al. 2019). FapA is recruited to the cell pole by the polar landmark protein HubP, which is very important for the formation of flagellum and bacterial motility. The interaction between dephosphorylated $EIIA^{Glc}$ and FapA inhibits the polar localization of FapA, resulting in suppression of flagellum formation. This process allows the bacteria to stay in a glucose-rich environment.

PTS is also involved in the regulation of biofilm formation. Recently, several studies have suggested the interaction between PTS system and biofilm formation in *Vibrio cholerae*. The deletion of PTS components, specifically EI, HPr, and $EIIA^{Glc}$ has affected the amount of biofilm formation (Houot, et al. 2010). $EIIA^{Glc}$ has been also found to interacts with MshH, which repesses biofilm formation (Pickering, et al. 2012).

3. The aim of this study

Quorum sensing is important for bacterial pathogenicity and biofilm formation. Although the VqmA-VqmR system plays an important role in pathogenicity and adaptation to the surrounding environment, their studies have been conducted only in *Vibrio cholerae*. The goal of this study is to elucidate the function of the VqmA-VqmR system during the infection process in *Vibrio vulnificus*. I aim to find out that *Vibrio vulnificus* has a VqmA-VqmR quorum sensing system and recognizes glucose-derived host signals.

Chapter II. Host-derived glucose activates quorum sensing signals and the virulence of *Vibrio vulnificus* during their infection

1. Introduction

To efficiently respond and adapt to environmental fluctuations, bacteria often exhibits the collective behaviors through an intercellular communication, referred to quorum sensing (QS) (Papenfort and Bassler 2016). The QS systems, relying on the production, sensing, responding to extracellular signaling molecules called autoinducers (AIs), induce population-wide changes in bacterial behaviors encompassing biofilm formation, production and secretion of toxins. Especially, in pathogenic bacteria, these systems play a crucial role in the regulation of virulence during infection. Owing to their importance, bacteria have evolved to preserve several distinct QS systems, which cooperatively control a variety of cellular responses (Papenfort and Bassler 2016; Ball, et al. 2017; Herzog, et al. 2019).

In pathogenic *Vibrio* species including *V. cholerae*, several distinct QS systems have been identified. *V. cholerae* has two canonical autoinducer molecules, CAI-1 ((S)-3-hydroxytridecan-4-one) and AI-2 ((2S,4S)2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate). their cognate membrane receptors are CqsS and LuxPQ, respectively (Jung, et al. 2015; Papenfort, et al. 2015). *Vibrio vulnificus* has AI-2-LuxPQ QS system (McDougald, et al. 2001; Li and Wang 2020). Despite the diversity of signaling molecules and receptors, the quorum sensing pathway in *vibrio* species is mostly mediated by LuxU-LuxO-Qrr small RNA (sRNA) system (Barrasso, et al. 2020). However, recently, VqmA-VqmR QS system has been newly identified (Papenfort, et al. 2015). VqmA is a cytoplasmic transcription factor that binds to autoinducer 3,5-dimethylpyrazin-2-ol (DPO) (Papenfort, et al. 2017). DPO is made from threonine and alanine, and its synthesis depends on threonine dehydrogenase (Tdh). The VqmA–DPO complex activates expression of *vqmR*, which encodes a small regulatory RNA. VqmR represses genes required for biofilm formation and virulence (Herzog, et al. 2019). *vqmA* and *vqmR* genes are also conserved in other *Vibrio* species, but few studies have

been conducted other than *V. cholerae*.

In addition to their cognitive autoinducers, several QS systems have been reported to be regulated by environmental signals. In *Escherichia coli*, AI-2 kinase LsrK interacts with HPr, a component of phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS), allowing bacteria to rapidly respond to changing nutrient levels at the population scale (Ha, et al. 2018). VqmA can detect the lack of oxygen and the presence of bile salts in *V. cholerae* (Mashruwala and Bassler 2020). Detection occurs via oxygen-, bile salt-, and redox-responsive disulfide bonds that alter VqmA DNA binding activity, allowing VqmA can integrate cell density, the presence or absence of oxygen, and host cues.

Despite of its importance in the physiological regulation under environmental stresses, few has been identified about the regulatory role of VqmA-VqmR system in *Vibrio* species other than *V. cholerae*. Here, I identified that VqmA-VqmR QS system plays a pivotal role in the regulation of virulence of *V. vulnificus*. Also, I found that this QS system is activated by glucose which acts as a host-derived signal that *V. vulnificus* encounter during their infection in the host blood vessel. I demonstrated that HPr interacts with VqmA and thus activates the transcriptional activity of VqmA in the presence of glucose. Finally, I found that the interaction between VqmA and HPr is highly species-specific because of the structural features of VqmA.

2. Materials and Methods

2.1. Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Construction of the deletion strains was performed as described previously. *V. vulnficus* strains were cultured in LBS medium (Luria-Bertani medium supplemented with additional 1% sodium chloride).

2.2. β-galactosidase reporter assay

A *V. vulnificus* MO6-24/O strain and its isogenic *vqmA*- and *tdh*-deleted mutants were transformed with the pJK1113-based plasmid carrying *E. coli* lacZ transcriptionally fused with *vqmR* promoter. Cells (80 μl) grown in the indicated conditions were 10-fold diluted with Z-buffer (100 mM sodium phosphate (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$ and 40 mM β-mercaptoethanol) and lysed using 20 μl 0.1% SDS and 40 μl chloroform. Then, using ortho-nitrophenyl b-galactoside (ONPG) as a substrate, the β-galactosidase activities of lysates were measured by monitoring optical density at 420 nm (Park, et al. 2013).

2.3. Survival test of *V. vulnificus*-infected mouse

Overnight-grown *V. vulnificus* MO6-24/O were inoculated in LBS medium and subsequently harvested when bacterial growth reached exponential phase and washed with PBS. Mice were deprived of food and water for 36 h before administration of *V. vulnificus*. Then, mice were intraperitoneally injected with 100 μl of PBS containing *V. vulnificus*. Mice were monitored every hour for 12 h after injection.

2.4. Purification of overexpressed proteins

VqmA and its variants were expressed in a *tdh*-deleted *E. coli* ER2566 strain, while EI and HPr were expressed in a *ptsHIcrr*-deleted ER2566 strain by adding 1 mM IPTG. Cells were resuspended with buffer A (25 mM HEPES-NaOH (pH 7.6), containing 100 mM NaCl) and disrupted by three passages through a French pressure cell at 8,000 psi. Then, hexahistidine-tagged proteins were purified using TALON metal-affinity resin (Takara Bio.) according to the manufacturer's instructions. To further purify those proteins, eluates were subjected Size exclusion chromatography using a Hiload 16/60 Superdex 200 pg column (GE Healthcare) equilibrated with buffer A.

2.5. Ligand fishing using metal affinity beads

Ligand-fishing experiments were performed as described previously to find a regulator of VqmA. *V. vulnificus* MO6-24/O cells overnight-grown at 37 ˚C at LBS supplemented with 0.1% glucose (200 ml) were harvested and resuspended in buffer A. Cells were then disrupted using sonicator for 1 h. Disrupted cells were centrifuged at 10,000 x g for 20 min at 4 ˚C, and the supernatant was mixed with 120 μg of HisVqmA or buffer A as control in the presence of TALON metal affinity resin in a 1.6 ml tube. After several washes with wash buffer (buffer A containing 10 mM imidazole), the proteins were eluted with elution buffer (buffer A containing 200 mM imidazole) and analyzed by SDS-PAGE, followed by staining with Coomassie brilliant blue R. Protein bands specifically enriched in the fraction with HisVqmA were excised from the gel, and in-gel tryptic digestion and peptide mapping using MALDI-TOF MS were carried out for protein identification.

2.6. Electrophoretic mobility shift assay (EMSA)

Nonradiolabeled EMSA was performed to dissect the DNA binding of VqmA. A 30-bp probe (~ 47 nM) were incubated with HisVqmA or its variant in the presence or absence of the effectors (DPO or HPr) in EMSA buffer (10 mM Tris–HCl (pH 8.0), 5% (v/v) glycerol, 0.1 mM EDTA and 1 mM DTT) with 200 μg/ml bovine serum albumin as non-specific competitor. Each mixture was incubated at 37 ˚C for 10 min and then electrophoresed on a 18% polyacrylamide gel (acrylamide/bisacrylamide ratio of 29:1) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) followed by staining with Ethidium bromide (EtBr). DNA probes were visualized and their intensities were quantified using ImageJ software

2.7. Microscale thermophoresis (MST) analysis

The binding affinities of VqmA with effectors (DPO and HPr) were measured using a NanoTemper Monolith NT.115^{pico} instrument. Purified HisVqmA was labelled using a Monolith protein labeling kit and used at a concentration of 5 nM. Each unlabeled DPO and HPr was titrated in 1:1 serial dilution in MST binding buffer (25 mM HEPES-NaOH (pH 7.6), 100 mM NaCl, 0.5 mg/ml BSA, 0.05 (v/v) % Tween 20), with the highest concentration of DPO at 250 μM and HPr at 25 μM. To assess the dependence of each effector on binding to VqmA, the binding affinity of one effector to VqmA was measured in the absence and presence of an excess amounts of the other effector. The measurements were performed at 20% LED power and 30% MST power at 22 ˚C.

2.8. Quantification of VqmA protein level by western blot

A *V. vulnificus* strain in which the chromosomal VqmA was tagged with Myc

at its C-terminus was grown in the indicated medium and harvested when OD600 reached 1.0. The pellets were resuspended in SDS sample buffer, and then cells were lysed at 100 ˚C for 5 min. Cell lysates were electrophoresed on an SDS-PAGE gel and VqmA was visualized using monoclonal mouse anti-Myc antibody (abcam).

Table 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Genotype or phenotype	Source
Strains		
Vibrio vulnificus		
MO6-24/O	Wildtype, Clinical isolate	
MO6-24/O Δ*vqmA*	MO6-24/O with Δ*vqmA*	This study
MO6-24/O VqmA::myc	MO6-24/O in which the chromosomal VqmA was tagged with myc at its c-terminus	This study
MO6-24/O Δ*tdh*	MO6-24/O with Δ*tdh*	This study
Escherichia coli		
ER2566	F^- l^- *fhuA2* [lon] *ompT lacZ*::*T7* gene 1 *gal sulA11* Δ(*mcrC*-mrr)114::IS10 R(*mcr*-73::miniTn10-TetS)2 R(*zgb*-210::Tn10)(TetS) *endA1* [*dcm*]	New England Biolabs
ER2566 Δ*tdh*	ER2566 with Δ*tdh*	This study
ER2566 Δ*ptsHIcrr*	ER2566 with Δ*ptsHIcrr*	This study
SM10 λ*pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*, OriT of RP4, Km^r; conjugational donor	(Miller *et al.*,1988)
Plasmids		
pDM4	Suicide vector for homologous recombination into *V. vulnificus* chromosome, OriR6K, Cm^r	(Milton *et al.*, 1996)
pETDuet-1		Novagen

pJK1113	pBAD24 with *oriT* of RP4 and *nptI*, P_{BAD}; Km^r , Amp^r	(Lim *et al.*, 2014)
pBAD-MycHisA	Broad host range vector, IncP ori, oriT of RK2, P_{lac}; Tc^r	Invitrogen
pRK415	Shuttle vector, *mob*$^+$, Tc^r	(Keen et al., 1988)
pDM4-*vqmA*	pDM4-based suicide vector for deletion of *vqmA*, Cm^r	This Study
pDM4-*tdh*	pDM4-based suicide vector for deletion of *tdh*, Cm^r	This Study
pET-HisVqmA	pETDuet-1-based expression vector for HisVqmA, Amp^r	This Study
pET-VqmA	pETDuet-1-based expression vector for VqmA, Amp^r	This Study
pET-HisHPr	pETDuet-1-based expression vector for HisHPr, Amp^r	This Study
pET-HPr	pETDuet-1-based expression vector for HPr, Amp^r	This Study
pBAD-VqmA	pBAD-MycHisA-based expression vector for VqmA, Amp^r	This Study
pJK1113-P_{vqmR}::*lacZ*	pJK1113-based expression vector for *E. coli* lacZ transcriptionally fused with *vqmR* promoter, Amp^r, Km^r	This Study
pRK415-HPr	pRK415-based expression vector for HPr	This Study
pRK415-HPr(H15A)	pRK415-based expression vector for HPr(H15A)	This Study

3. Results

3.1. Glucose activates VqmA-VqmR QS system.

VqmA-VqmR system is a highly conserved quorum-sensing (QS) signal system among *Vibrio* species (Papenfort, et al. 2015). In *Vibrio vulnificus* MO6-24/O, it consists of transcription factor VqmA which encoded by VVMO6_04512, and its cognitive cis-encoded sRNA VqmR, which is located upstream of VqmA (Figure 1a). Using a *vqmR*::*lacZ* transcriptional fusion construct, I found that VqmA activates the expression of *vqmR* in *V. vulnificus*, consistently with the previous results in *V. cholerae* (Papenfort, et al. 2015), and confirmed that this signal system is active in the species (Figure 1a). As QS signaling plays a pivotal role in the virulence in pathogenic *Vibrio* species during their host infection (Kim, et al. 2003; Zhu and Mekalanos 2003; Kamareddine, et al. 2018), I assessed the regulatory role of VqmA in the pathogenicity of *V. vulnificus* by comparing the survival rate of mice which were intraperitoneally injected by either wild-type or its isogenic *vqmA* deletion mutant (Δ*vqmA*) *V. vulnificus* MO6-24/O cells. I observed the significantly increased survival rate of mice infected by Δ*vqmA* cells compared to those infected by wild-type cells, and confirmed that VqmA activates the virulence of *V. vulnificus* upon infection (Figure 1b).

A previous study in *V. cholerae* has reported that VqmA senses a variety of host-derived signals including anaerobic condition and bile salts that V. *cholerae* can encounter in the host gut during their infection and integrates these signals with QS signal by regulating its transcriptional activity via an intramolecular disulfide bond between Cys48 and Cys63 (Mashruwala and Bassler 2020). Interestingly, in VqmA of *V. vulnificus* MO6-24/O, Cys63 was substituted with Phenylalanine (Figure 2). Also. I was not able to find any change in the VqmA activity depending on the concentration of oxygen or the

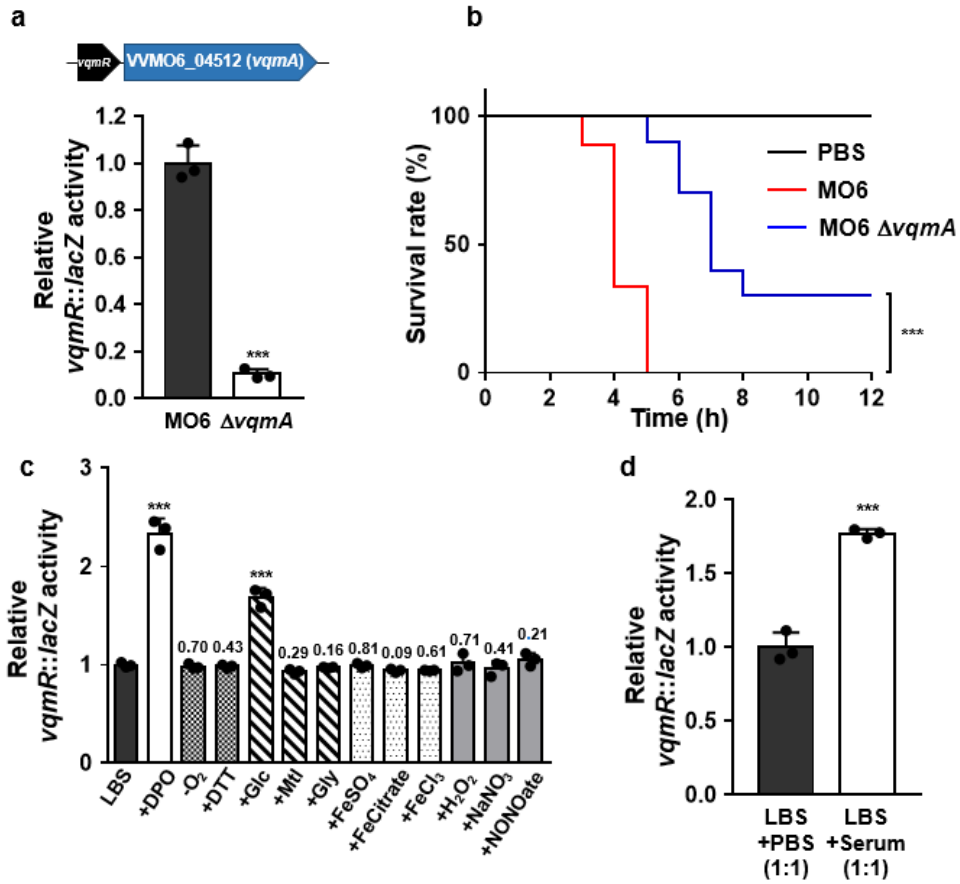

Figure 1. Glucose-responsive VqmA activity in *Vibrio vulnificus*.

a, Schematic of the *V. vulnificus vqmA* and *vqmR* genomic locus and transcriptional activity of VqmA. *vqmR* expression level was measured using β-galactosidase assay in Wild-type and Δ*vqmA V. vulnificus* strains. **b**, Survival test of *V. vulnificus*-infected mouse. Viability of mouse in the absence of bacteria or following challenge by WT or Δ*vqmA V. vulnificus*. mice were intraperitoneally injected with PBS containing *V. vulnificus*. **c** and **d**, *vqmR* expression level was measured using β-galactosidase assay with indicated condition. Each final concentration is 500 μM for DTT, 0.1% for sugars, 30 μM for iron compound, and 0.2 mM for reactive oxygen species or reactive nitrogen species. Glc, glucose; Mtl, mannitol; Gly, glycerol.

Statistical significance was assessed using Student's t-test (β-galactosidase assay) and log rank test (mouse infection assay) (***p value <0.001, p values greater than 0.01 were presented). Shown are the means and SD (n=3, independent measurements).

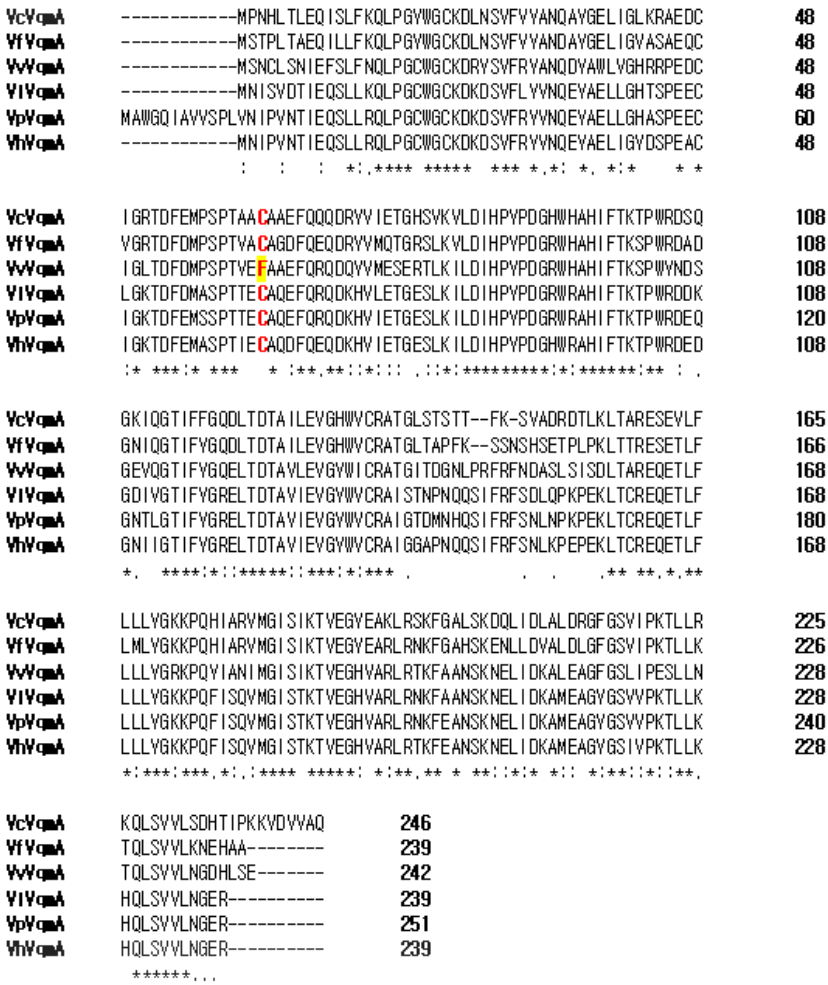

Figure 2. VqmA of *V. vulnificus* has species-specific phenylalanine residue.

Alignment of amino acid sequences of VqmA homologs from *Vibrio* species using ClustalW software. Identical (*), strongly similar (:), and weakly similar (.) residues are indicated. The 63rd amino acid of each VqmA is colored in red. in VqmA of *V. vulnificus* MO6-24/O, Cys63 was substituted with Phenylalanine. Vc, *Vibrio cholerae*; Vf, *Vibrio furnissii*; Vv, *Vibrio vulnificus*; Vl, *Vibrio alginolyticus*; Vp, *Vibrio parahaemolyticus*; Vh, *Vibrio harveyi*.

presence of reducing agent Dithiothreitol (DTT) (Fig. 1c), which affected the transcriptional activity of *V. cholerae* VqmA (Mashruwala and Bassler 2020). Then, I wondered if VqmA would sense another signal that is specific to infection niche for *V. vulnificus*. As *V. vulnificus* infection causes severe sepsis accompanied with liver diseases including cirrhosis or hepatitis (Strom and Paranjpye 2000; Bross, et al. 2007), I assumed that any signal molecule in host blood or these organs could act as a regulator of VqmA that enables *V. vulnificus* distinguishes its host environments from its natural environment. As expected, I observed the increased expression of *vqmR* in cells cultured in the medium supplemented with 50% (v/v) serum which was obtained from mice blood (Fig. 1d).

Then, I screened the potential regulator of VqmA among several signals including carbon sources, iron, and reactive oxygen species or reactive nitrogen species that *V. vulnificus* might encounter in the host blood (Fig. 1c). Among them, glucose was the only signal that induced the expression of *vqmR*. While estuarine seawater, the natural habitats of *V. vulnificus*, are usually oligotrophic and thus deprived of glucose, bacterial cells upon infection are exposed to millimolar levels of glucose in the blood vessel (Kim, et al. 2010). Thus, I speculated that glucose might be the signals that *V. vulnificus* recognize the host environment. When I measured the *vqmR* expression in the presence of the increasing amounts of glucose from 0.01% to 0.5% in the medium, I found that this QS system could respond to glucose at the minimum concentration of 0.05% (Figure 3a). Considering that the concentration of glucose in mouse or human blood is approximately 0.1% or above, these results supported the notion that glucose could act as a regulator of VqmA-VqmR QS system during the infection of *V. vulnificus* in the mammalian host. To investigate the underlying mechanism how glucose activates the VqmA-VqmR QS signaling system, I tested three possibilities;

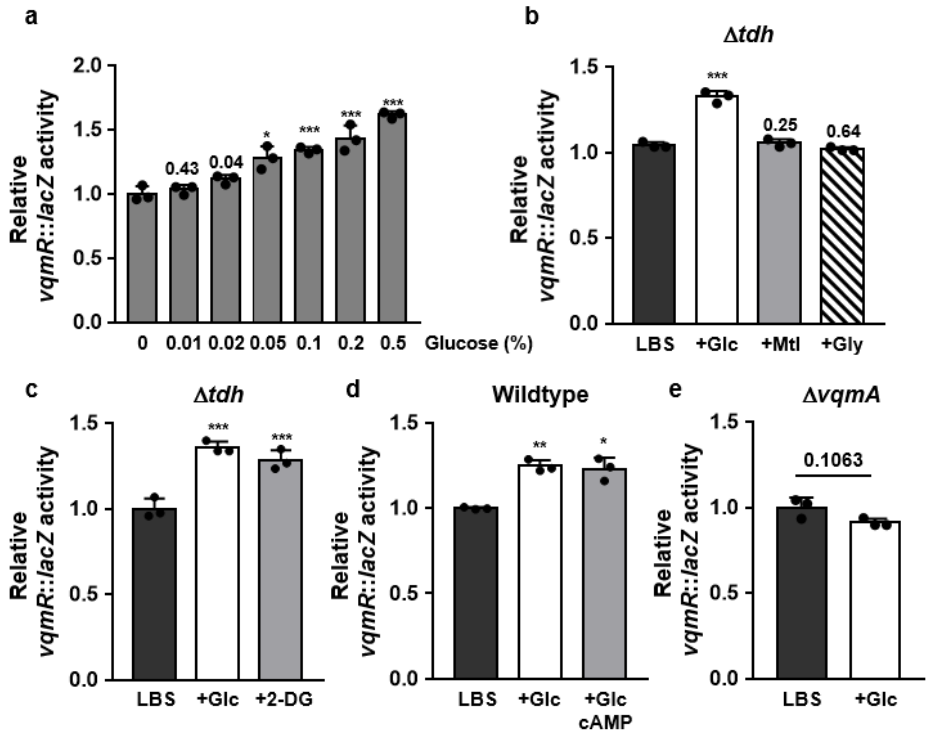

Figure 3. Increased *vqmR* expression level by glucose is not due to DPO, metabolite effector, or cAMP.

a, The expression level of *vqmR* was measured using β-galactosidase assay in Wild-type *V. vulnificus* with increasing amounts of glucose (0- 0.5%)**. b~e,** the *vqmR* expression level was measured using β-galactosidase assay in Wild-type, Δ*tdh* and Δ*vqmA V. vulnificus* strains. Indicated sugars were added at 0.1% concentration. Glc, glucose; Mtl, mannitol; Gly, glycerol; 2-DG, 2-deoxyglucose. cAMP was added at 5 mM concentration. Statistical significance was assessed using Student's t-test (**p* value <0.01, ***p* value <0.005, ****p* value <0.001, *p* values greater than 0.01 were presented). Shown are the means and SD (n=3, independent measurements).

(i) glucose activates the production of auotuinducer DPO or other unidentified metabolite effector (ii) glucose indirectly regulates the expression of *vqmR* not via VqmA (iii) glucose regulates the expression or activity of VqmA. To test whether glucose controls the QS system through regulating the level of DPO, I measured the *vqmR* expression in Δ*tdh* mutant, which has been reported to be unable to produce DPO (Papenfort, et al. 2017), in the absence and presence of glucose (Figure 3b). However, glucose still activated the *vqmR* expression. Also, I observed that the addition of 2-deoxyglucose (2-DG), non-metabolized glucose analog (Wick, et al. 1957), induced the *vqmR* expression, suggesting that any metabolite derived from glucose metabolism was not the effector of VqmA (Figure 3c). These results concluded that glucose-dependent activation of VqmA-VqmR system is not through the increasing production of DPO or another metabolite effector. Then, I tested whether glucose indirectly regulates the *vqmR* expression. In various bacterial species including *E. coli* and *V. vulnificus*, it has been shown that the level of cAMP decreases in the presence of glucose and the global transcription regulator CRP, complexed with cAMP, is involved in the glucose-dependent transcriptional regulation of more than hundreds of genes (Kimata, et al. 1997; Park, et al. 2006; Gorke and Stulke 2008). However, I could find no difference in the *vqmR* expression when I exogenously added cAMP in the LBS medium supplemented with glucose (Figure 3d). Also, glucose was not able to induce the *vqmR* expression in the *vqmA* deletion mutant, showing that glucose regulates the QS signals through VqmA. Finally, to test whether the expression or activity of VqmA was affected by glucose, I firstly measured the protein level of VqmA in the cells cultured in the LBS medium supplemented with either glucose, mannitol or glycerol using western blot experiments (Figure 4). However, it had similar levels among those conditions and I collectively concluded that there is other regulator(s) of the

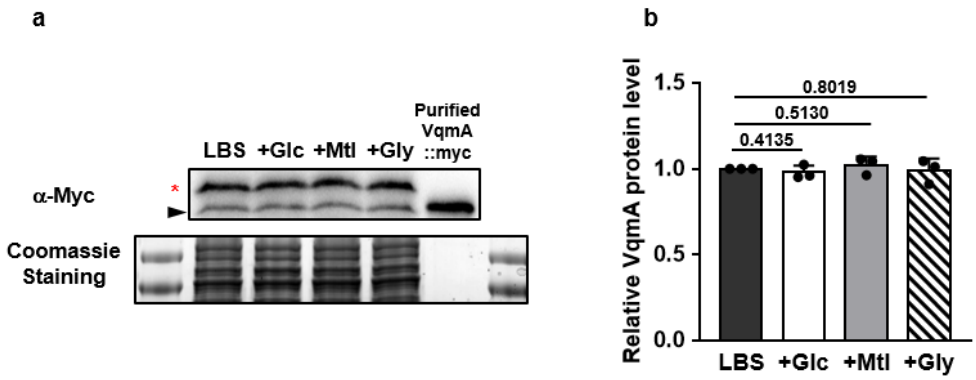

Figure 4. The protein expression level of VqmA is not affected by sugars.

a, The protein expression level of VqmA was measured using western blot experiments. A *V. vulnificus* strain in which the chromosomal VqmA was tagged with Myc at its C-terminus was grown in the indicated medium and harvested when OD600 reached 1.0. Cell lysates were electrophoresed on an SDS-PAGE gel and VqmA was visualized using monoclonal mouse anti-Myc antibody. **b**, Relative VqmA protein levels from the same samples presented in panel **a**. Data were normalized to the level of VqmA-FLAG in panel **a**. Statistical significance was assessed using Student's t-test (*p* values greater than 0.01 were presented). Shown are the means and SD (n=3, independent measurements).

transcriptional activity of VqmA that is activated in the cells growing in the glucose medium.

3.2. Dephosphorylated HPr increases the transcriptional activity of VqmA

To find any regulating protein that interacts with VqmA in the presence of glucose, I performed ligand fishing experiments using purified hexahistidine-tagged VqmA (HisVqmA) as a bait (Figure 5a) (Heo, et al. 2019). Crude extracts prepared from *V. vulnificus* MO6-24/O cells grown in LBS medium supplemented with 0.1% glucose were mixed with TALON affinity resin in the presence and absence of HisVqmA. After several washes with 10 mM imidazole, protein pools eluted with 200 mM imidazole were subjected to SDS-PAGE followed by staining with Coomassie brilliant Blue R. Repeated analyses revealed dramatic enrichment of a protein band with molecular mass of approximately 10 kDa in the fraction with HisVqmA. In-gel tryptic digestion and peptide mapping identified that this band correspond to HPr, a component of phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS), which is encoded by VVMO6_02248. PTS is a multi-component carbohydrate translocation system that is involved in the transport of more than 20 carbohydrates in most bacteria (Deutscher, et al. 2006). In addition to its transport activities, it has been well studied that PTS components including HPr, interact with numerous proteins and thus regulate functions of the interacting proteins, depending on their phosphorylation states that were determined by the availability of carbohydrates (Deutscher, et al. 2006). Thus, I speculated that HPr is a feasible regulator of VqmA that increases the *vqmR* expression in the presence of glucose.

To verify the specific interaction between VqmA and HPr, I performed a reciprocal ligand fishing experiment using purified HisHPr as a bait

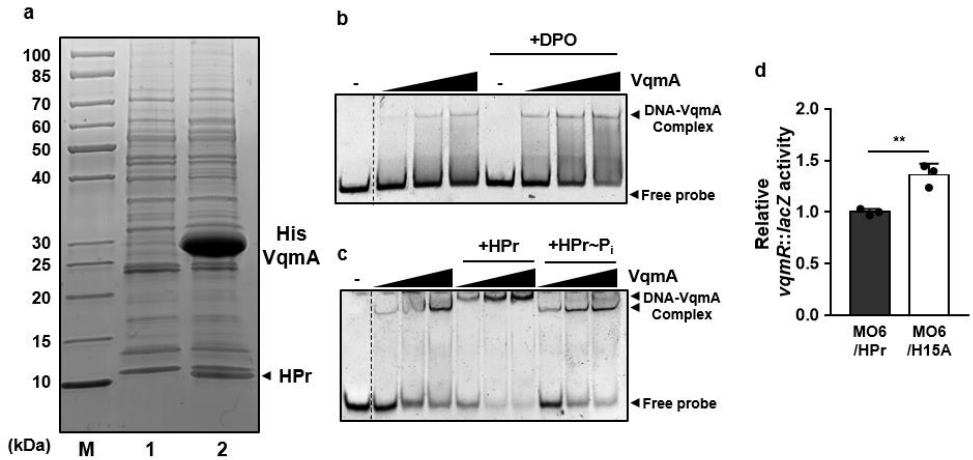

Figure 5. Dephosphorylated HPr increases the transcriptional activity of VqmA

a, Ligand fishing experiment was carried out to find proteins interacting with HisVqmA. Crude extract prepared from *V. vulnificus* MO6-24/O cells was mixed with buffer A (lane 1) or 120 μg of purified HisVqmA (lane 2). Each mixture was subjected to TALON metal affinity chromatography and proteins bound to the column were analyzed as described in Materials and Methods. **b** and **c**, VqmA binding to *vqmR* promoter region was examined using electrophoretic mobility shift assay (EMSA). HPr, HPr+EI, or DPO was added to find the effect of dephosphorylated HPr, phosphorylated HPr, DPO on the transcriptional activity of VqmA respectively. **d**, The expression level of *vqmR* was measured using β-galactosidase assay in wild-type strain carrying an expression vector for wild-type HPr and the wild-type strain carrying an expression vector for dephosphomimetic mutant (H15A) of HPr**.** Statistical significance was assessed using Student's t-test (***p* value <0.005,). Shown are the means and SD (n=3, independent measurements).

(Figure 6a). Cell lysates of *V. vulnificus* MO6-24/O were incubated with TALON metal affinity resin in the absence and presence of HisHPr. To dissect the phosphorylation state-dependent interaction of HPr with VqmA, HPr was dephosphorylated by adding pyruvate or phosphorylated by adding PEP to the mixture (Lee, et al. 2019). I observed the protein band corresponded to Pyruvate kinase PykA, which has been reported to interact only with dephosphorylated HPr in *V. vulnificus* (Kim, et al. 2015), was co-eluted with only dephosphorylated HPr, validating the experiment. Then, I found VqmA protein band was co-eluted with HisHPr, yet in the set incubated with PEP, showing that VqmA interacts with only dephosphorylated HPr.

Further, I performed pull-down experiment using purified HisVqmA and untagged HPr (Figure 6b). Firstly, I modulated the phosphorylation state of HPr by incubating with EI in the absence or presence of PEP as described previously (Choe, et al. 2017). Then, dephosphorylated and phosphorylated HPr was mixed with increasing amount of purified HisVqmA and subjected to TALON metal affinity chromatography. I found that HPr interacted with HisVqmA in the VqmA concentration-dependent manner in the absence of PEP, but no HPr was co-eluted with HisVqmA in the presence of PEP. Collectively, these results concluded that only dephosphorylated HPr interacts with VqmA.

To assess the effect of HPr on the function of VqmA, I firstly measured the DNA binding affinity of VqmA in the absence and presence of HPr using electrophoresis mobility shift assay (EMSA) (Figure 5b and 5c,). In *V. cholerae*, binding of autoinducer DPO to VqmA slightly resulted in the increases of the DNA binding affinity and transcriptional activity (Huang, et al. 2020). Consistently, I found that the slightly increased DNA binding affinity of VqmA in the presence of DPO (Figure 5b). Then, I observed that

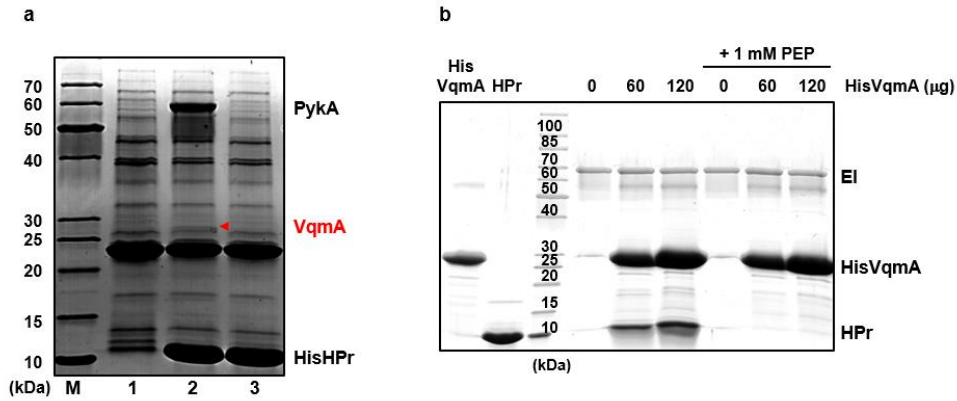

Figure 6. VqmA interacts with only dephosphorylated HPr.

a, Reciprocal ligand fishing experiment using purified HisHPr as a bait was performed. Crude extract prepared from *V. vulnificus* MO6-24/O cells was mixed with buffer A (lane 1) or 120 μg of purified HisHPr (lanes 2 and 3). 1 mM pyruvate (lane 2) or 1 mM PEP (lane 3) was added to the mixture. Each mixture was subjected to TALON metal affinity chromatography and proteins bound to the column were analyzed as described in Materials and Methods. **b**, Pull-down experiment using purified HisVqmA and untagged HPr was performed. 1 mM PEP and EI were added to phosphorylate HPr.

DNA binding of VqmA was enhanced with a 6-fold higher affinity than the ligand-free form, whereas there was no difference in binding affinity when phosphorylated HPr was added (Figure 5c). Then, to verify whether dephosphorylated HPr enhances the transcriptional activity of VqmA in the cells, I compared the *vqmR* expression of the wild-type strain carrying an expression vector for wild-type HPr with the wild-type strain carrying an expression vector for dephosphomimetic mutant (H15A) of HPr, and observed increased expression of *vqmR* in the latter strain (Figure 5d). When I determined the phosphorylation state of HPr in the LBS medium supplemented with several sugars, I found that only in the presence of glucose, HPr was thoroughly dephosphorylated (Figure 7). Together, our data show that dephosphorylated HPr activates VqmA in the presence of glucose. When I measured the binding affinities of DPO and HPr to VqmA, the presence of one merely affected on the binding of the other (Figure 8). Thus, I concluded that HPr-mediated activation of VqmA is independent on the DPO.

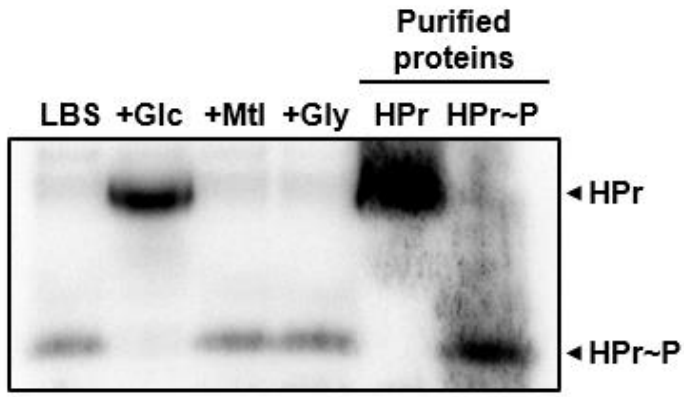

Figure 7. HPr is dephosphorylated in the presence of glucose.

The phosphorylation state of HPr was determined by western blot using anti-HPr mouse serum after SDS-PAGE in the presence of various carbon sources. HPr is dephosphorylated only in the presence of glucose.

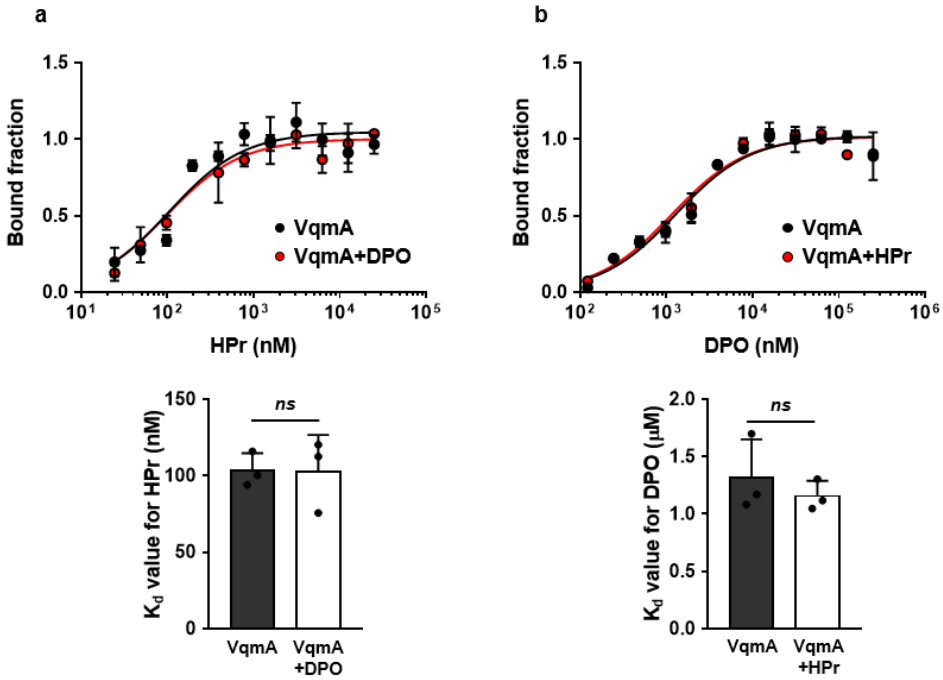

Figure 8. HPr-mediated activation of VqmA is independent on the DPO.

The binding affinities of VqmA with HPr (**a**) and DPO (**b**) were measured using a NanoTemper Monolith NT.115pico instrument. The dissociation constants (K_d) of HPr and DPO complexed with VqmA were obtained from three technical replicates. Shown are the means and SD (n=3, independent measurements).

3.3. Interaction between HPr and VqmA is highly species-specific.

Intriguingly, the interaction between HPr and VqmA was not conserved in *V. cholerae* (Figure 9a). To test whether the interaction between two proteins is specific in *V. vulnificus*, I purified HisVqmA and HPr proteins from *V. vulnificus* and *V. cholerae* and tested cross-interactions between these proteins. Whereas *Vv*VqmA interacted with *Vc*HPr as well as *Vv*HPr, *Vc*VqmA did not interact with both HPrs in the pull-down experiments. BLAST searches revealed that *Vv*VqmA shares only 62.76% amino acid sequence with *Vc*VqmA, while *Vv*HPr shares 96.47% with *Vc*HPr. Together, these data suggested that the interaction between HPr and VqmA is highly species-specific and is dependent on the surface structure of VqmA.

Analysis of amino acid sequences revealed that VqmA possesses a Per-Arnt-Sim 4 (PAS4) ligand-binding domain in its N-terminus (3-125 aa) and a helix-turn-helix (HTH) DNA-binding domain in its C-terminus (159-213 aa) (Huang, et al. 2020). To characterize molecular features of VqmA required for this protein-protein interaction, I constructed a series of chimeric mutants of VqmA through domain-swapping with *Vc*VqmA and tested the interaction of these mutants with HPr using in vitro binding assay (Figure 9b). I observed that only *Vv*VqmA (VcHTH) and *Vc*VqmA (VvPAS) that possess PAS4 domain of *V. vulnificus* VqmA interacted with HPr, confirming the indispensable role of PAS4 domain in the interaction between VqmA and HPr. Thus, I concluded that the interaction between VqmA and HPr is species-specific and is dependent on the structure of the PAS4 domain of VqmA.

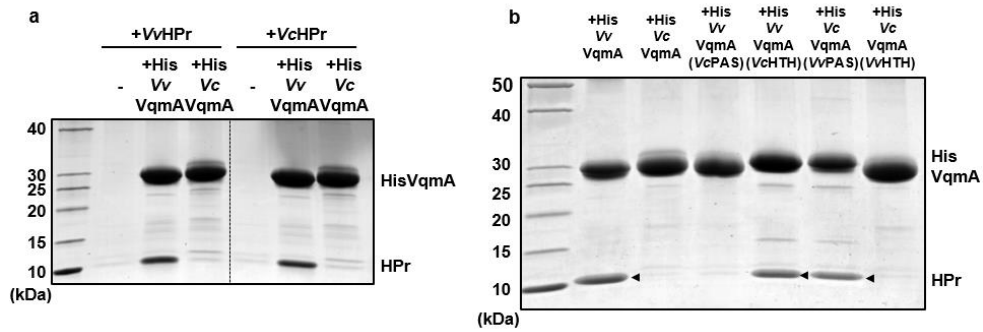

Figure 9. The interaction between HPr and VqmA is dependent on the surface structure of VqmA.

a. Pull-down experiment using purified HisVqmA and untagged HPr from *V. vulnificus* or *V. cholerae* was performed. HisVqmA from from *V. vulnificus* or *V. cholerae* was incubated with untagged HPr from *V. vulnificus* or *V. cholerae***.** Each mixture was subjected to TALON metal affinity chromatography. **b**, Pull-down experiment using purified HisVqmA or chimeric mutants of HisVqmA and untagged HPr from *V. vulnificus* or *V. cholerae* was performed. WT or chimeric mutants of HisVqmA was incubated with untagged VvHPr. Each mixture was subjected to TALON metal affinity chromatography.

4. Discussion

In this study, I identify the role of VqmA-VqmR quorum sensing system in *V. vulnificus*. VqmA activates the expression of *vqmR* sRNA and affects the virulence of *V. vulnificus* in the mouse infection model. The *vqmR* expression level is increased in the presence of glucose. In the presence of glucose, HPr is dephosphorylated, which can interact with VqmA. As the dephosphorylated HPr binds to VqmA, the transcriptional activity of VqmA is increased and the expression level of *vqmR* is increased. This interaction between HPr and VqmA is highly species-specific (figure 10).

The ability of bacteria to sense and respond to their environment is crucial to their survival (Brown and Williams 1985; Roszak and Colwell 1987). *V. vulnificus* is commonly found in marine environments and has been isolated from shellfish such as oysters (Morris 2003; Jones and Oliver 2009). *V. vulnificus* controls the biofilm, which plays an important role in the infection process, by recognizing the concentration of calcium in the oyster (Garrison-Schilling, et al. 2011; Park, Lee, et al. 2016; Chodur, et al. 2018; Pu, et al. 2020). *V. cholerae* recognizes the host-induced signals by modulating the activity of VqmA according to the bile salts concentration in the small intestine (Mashruwala and Bassler 2020). Thus, my finding that the activity of VqmA is affected by glucose means that *V. vulnficus* recognizes host-induced signals through glucose in the blood vessel.

Canonical LuxR-type QS receptors require binding of their cognate ligands for transcriptional regulation (Zhu and Winans 1999, 2001; Schuster, et al. 2004; Swem, et al. 2009). However, VqmA does not require DPO for basal transcriptional activity (Huang, et al. 2020). The fact that it has basal activity without DPO binding suggests that other regulators can affect VqmA in the absence of DPO. In the small intestine, the activity of VqmA is mainly

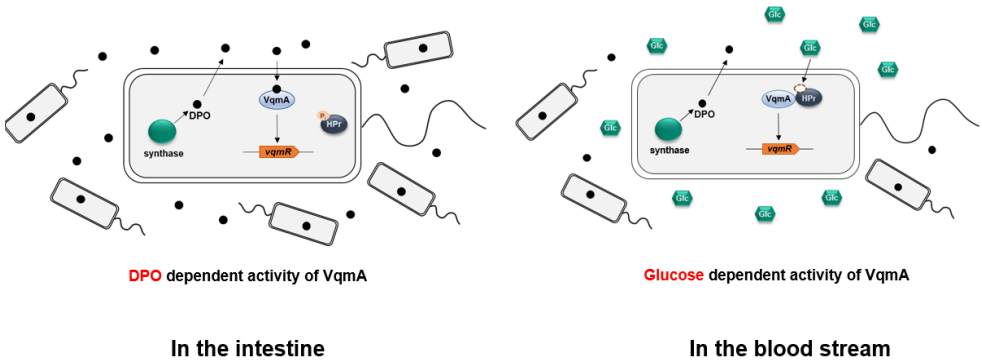

Figure 10. The activity of VqmA is regulated differently depending on the environment.

The small intestine is a DPO-rich environment because intestinal microbes carrying a *tdh* gene can secrete DPO. Thus, in the small intestine, the activity of VqmA is mainly dependent on DPO. However, the effect of DPO is less because there are fewer microorganisms in the blood and the blood flow rate is faster. Instead, *V. vulnificus* regulates VqmA activity while transporting glucose that is abundantly present in the blood. As glucose is transported through the glucose PTS, HPr becomes dephosphorylated and can bind to VqmA. As the dephosphorylated HPr binds to VqmA, the binding affinity of VqmA increases and the expression level of VqmR is increased.

affected by DPO secreted by many intestinal microbes. However, in blood, the effect of DPO will be less because of the absence of surrounding microorganisms and the high blood flow rate. Thus, in the blood, VqmA activity will be affected by glucose abundantly present in the blood instead of DPO to regulate pathogenicity.

VqmA can interact with HPr only in *V. vulnificus*, not in *V. cholerae* (Figure 9a). This is due to the structural difference between the PAS4 domains of *V. vulnificus* VmqA and *V. cholerae* VqmA (Figure 9b). Future efforts will focus on identifying the surface structural difference between the PAS4 domain of VqmA in *V. vulnificus* and *V. cholerae*. Also, the genes regulated by VqmR in *V. vulnificus* have not been identified. So, I'm trying to find glucose specific VqmR target genes based on RNA-sequencing.

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국문 초록

체내 감염 상황에서 비브리오 패혈증균의 혈당 인식에 따른 병원성 조절

박테리아는 다양한 세포 외 환경을 감지하며 이에 반응한다. 정족수 감지 기작은 박테리아가 주변 환경의 박테리아 세포 밀도와 종 구성의 변화를 감지하고 이에 반응하여 유전자를 조절하는 기작이다. 비브리오 콜레라균에서, VqmA-VqmR 정족수 감지 기작이 밝혀졌는데, 체내 감염상황에서 숙주의 환경을 인식하는 기능을 한다. 본 연구에서는 비브리오 패혈증균에서의 VqmA-VqmR 정족수 감지 기작의 기능을 밝히고자 한다. 전사인자인 VqmA는 *vqmR* 유전자 발현을 활성화시키고, 실제 쥐 감염 모델에서 병원성을 조절한다. 포도당이 존재할 때 vqmR의 발현량이 증가하는 것을 밝혔는데, 이는 포도당이 존재할 때 탈인산화 된 HPr이 VqmA와 결합하여 VqmA의 전사 활성도를 높였기 때문이다. 이러한 VqmA와 HPr의 결합은 종 특이적으로, 패혈증균에서만 보존이 되어있다. 즉 패혈증균은 VqmA-HPr 상호작용을 통해 체내 감염 상황에서 숙주 내 환경을 인지하고 병원성을 조절한다.

주요어:

정족수 감지, 당 수송 인산전달계, VqmA, VqmR, HPr, 포도당

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