Letter to the Editor

Characterization of the CpsQ Regulon Reveals Its Role in the Transcription of Type VI Secretion System 2 Genes in *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus, a gram-negative halophilic bacterium that naturally inhabits coastal waters, causes gastroenteritis, skin infections, and septicemia in human beings[1]. This bacterium produces multiple virulence factors, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), type III secretion system 1 (T3SS1), T3SS2, type VI secretion system 1 (T6SS1), and T6SS2[1]. Furthermore, *V. parahaemolyticus* forms biofilms on the surface, which help it in adapting to unfavorable conditions[2]. Mature biofilm formation requires special structures, including lateral and polar flagella, exopolysaccharide (EPS), and type IV pili[2].

CpsQ, a c-di-GMP-binding regulatory protein, is encoded by the *cpsQ-mfpABC* operon[3]. Although CpsQ plays important roles in biofilm formation and the expression of RTX matrix proteins and capsules[4], whether it can regulate other genes remains obscure. In this study, nucleotides 35 to 616 of *cpsQ* were deleted from the genome of *V. parahaemolyticus* RIMD2210633 (wild-type, WT) using the suicide plasmid pDS132 to generate the *cpsQ* deletion mutant (*ΔcpsQ*)[5], and then the RNA sequencing (RNA-seq) and several phenotypic and molecular experimental assays were employed to explore the genes regulated by CpsQ.

Overnight cell cultures of WT and *ΔcpsQ* in 2.5% (w/v) Bacto heart infusion (HI) broth (BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C were serially diluted 10-fold using phosphate-buffered saline (PBS) buffer (pH 7.2); then, 200 μL of the diluted cells was spread onto an HI plate, followed by statical incubation at 37 °C for 48 h. Colonies were randomly selected in triplicate for each strain, resuspended in PBS, and adjusted to obtain an OD_{600} value of 1.4 for each bacterial suspension (defined here as bacterial seeds); they were then diluted 50-fold using 10 mL HI broth and allowed to continuously grow at 37 °C with shaking at 200 rpm. The OD_{600} values of all cultures were measured at 1 h intervals to plot growth curves. As shown in Supplementary Figure S1, available in www.besjournal.com, the two strains showed similar growth rates under the studied growth conditions, which suggested that CpsQ did not regulate the growth of *V. parahaemolyticus*.

Two colonies of each strain were randomly harvested from the HI plate and placed in TRIzol reagent (Invitrogen, California, USA) for RNA extraction. RNA-related operations were performed at GENEWIZ Biotechnology Co., Ltd. (Suzhou, China). The mRNA profiles in *ΔcpsQ* (test group) were compared with those of WT (reference group) *via* RNA-seq to determine the genes that were regulated by CpsQ in *V. parahaemolyticus*. Significantly differentially expressed genes (DEGs) were analyzed using the DESeq2 (V1.6.3) software with at least two-fold changes in the ratio of mRNA levels (test/reference) and a *P*-value of < 0.05. As shown in Figure 1, CpsQ regulated the transcription of 567 genes, including 311 upregulated and 441 downregulated genes. Of these, 28 polar flagellar genes and 23 T6SS2 genes were down- and upregulated, respectively, in *ΔcpsQ* relative to those in WT (Supplementary Table S1, available in www.besjournal.com). A total of six genes encoding GGDEF- or EAL-domain proteins were significantly differentially expressed in *ΔcpsQ* relative to those in WT (Supplementary Table S1). Additionally, at least 31 putative regulatory genes, including 12 putative two-component system genes (highlighted with gray shadows in Supplementary Table S1), were significantly differentially expressed in *ΔcpsQ*

doi: 10.3967/bes2024.049

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relative to those in WT. Other genes such as antioxidative genes, T3SS2 genes, and putative outer membrane protein genes were also differentially expressed in *ΔcpsQ* and WT (Supplementary Table S1). The reliability of the transcriptome data was validated using quantitative PCR (qPCR) (Figure 2), which was performed as described previously[6]. The primers used in this study are listed in Supplementary Table S2, available in www.besjournal.com.

Approximately 50% of the polar flagellar genes were activated by CpsQ (Supplementary Table S1). Therefore, we investigated whether CpsQ regulates polar flagellum-mediated swimming motility. Two microliters of bacterial seeds were inoculated onto a semi-solid swimming plate containing 1% Oxoid tryptone (Oxoid, Basingstoke, England), 2% NaCl (Merck, Darmstadt, Germany), and 0.5% Difco Noble agar (BD Biosciences). The diameters of swimming areas were measured per hour after incubation at 37 °C. Results showed that the swimming capacities of WT and *ΔcpsQ* were not significantly different under the studied growth conditions at all time points tested (Supplementary Figure S2A, available in www.besjournal.com), which contradicted the results of RNA-seq and qPCR (Supplementary Table S1 and Figure 2). To verify whether the contradiction is attributed to the different growth conditions, *V. parahaemolyticus* strains were grown in the same medium as the swimming plate but with no agar at 37 °C and harvested at the mid-log phase. qPCR assays were employed to detect the regulation of polar flagellar genes (VP0785 and VP0788) by CpsQ. As shown in Supplementary Figure S2B, the mRNA levels of VP0785 and VP0788 were similar in *ΔcpsQ* compared to those in WT, which suggested that CpsQ had no regulatory effect on the transcription of polar flagellar genes. Therefore, CpsQ did not regulate the swimming motility of *V. parahaemolyticus*.

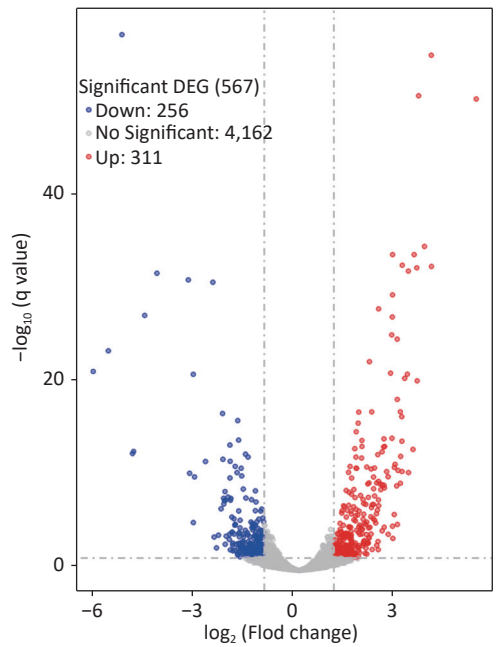

Figure 1. RNA-seq revealed the genes regulated by CpsQ on the HI agar plate. Volcano plot. Red, blue, and grey points indicate the upregulated, downregulated, and non-significant genes, respectively.

Six putative c-di-GMP metabolism-associated genes, including *tpdA*, are regulated by CpsQ (Supplementary Table S1). TpdA is a phosphodiesterase that plays a key role in the modulation of intracellular c-di-GMP levels, motility, and biofilm formation[7]. This prompted us to determine whether CpsQ regulates c-di-GMP production. The WT and *ΔcpsQ* colonies were

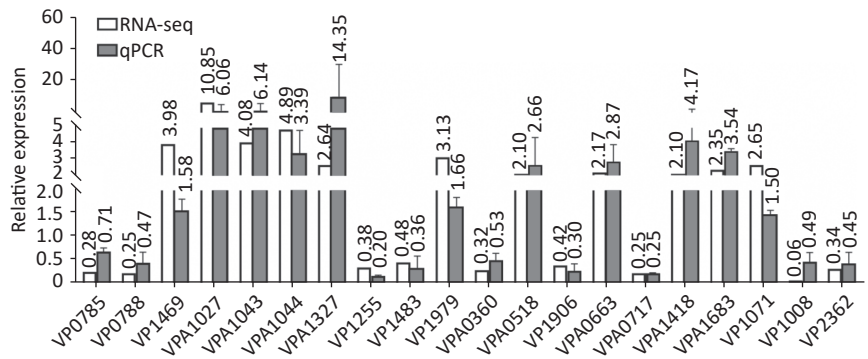

Figure 2. The transcriptional variation between *ΔcpsQ* (test) and WT (reference) was calculated for each target gene. A mean ratio of 2 was designated as the cutoff for determining statistical significance. The 16S rRNA gene was used as the internal control. WT, wild type.

randomly collected in triplicate from the HI plate, resuspended in 2 mL ice-cold PBS (pH 7.2), incubated at 100 °C for 5 min, and sonicated for 15 min (power, 100%; frequency, 37 kHz) in an ice-water bath. The intracellular c-di-GMP levels and total protein in the supernatants were measured using a c-di-GMP enzyme-linked immunosorbent assay (ELISA) kit (Mskbio, Wuhan, China) and Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Massachusetts, USA), respectively. Intracellular c-di-GMP levels are expressed as picomoles per gram of protein. As shown in Supplementary Figure S3 (available in www.besjournal.com), there were no significant differences in the intracellular c-di-GMP levels between WT and *ΔcpsQ*, suggesting that CpsQ did not affect the synthesis of c-di-GMP in *V. parahaemolyticus*.

RNA-seq and qPCR data revealed that CpsQ repressed the transcription of T6SS2 (Supplementary Table S1 and Figure 2) genes. The T6SS2 gene cluster (VPA1024-1046) contains three operons—VPA1027-1024, VPA1043-1028, and VPA1044-1046[8]. VPA1043-1028 and VPA1044-1046 are transcribed in opposite directions and are adjacent to each other[8]. Thus, the first genes, VPA1027-1024 and VPA1043-1028, were selected as target genes to further investigate CpsQ-mediated gene expression. The regulatory DNA region of each target gene was cloned into the pHRP309 vector harboring the promoter-less *lacZ* reporter gene. Each recombinant plasmid was transferred into WT and *ΔcpsQ*. Colonies of the resulting transformants were lysed to measure β-galactosidase activity in the cellular extracts using a β-Galactosidase Enzyme Assay System (Promega, Madison, USA)[9]. As shown in Figure 3A, the promoter activity (Miller units) of VPA1027 or VPA1043 in the *ΔcpsQ* colony was significantly enhanced relative to that in the WT colony, suggesting that CpsQ repressed the promoter activities of T6SS2 genes in *V. parahaemolyticus*. In addition, *E. coli* 100 λpir (Epicenter) bearing pBAD33-*cpsQ* or pBAD33 and a recombinant *lacZ* plasmid were cultured in Luria-Bertani broth containing 0.1% L-arabinose and 20 μg/mL chloramphenicol at 37 °C and harvested when an OD_{600} value of 1.2 was reached, to test whether CpsQ regulates the T6SS2 gene expression in a heterologous host[10]. As shown in Figure 3B, the expression of CpsQ from pBAD33-*cpsQ* led to much lower promoter activities of VPA1027 and VPA1043 compared to those in strains bearing pBAD33. These findings indicated that CpsQ directly regulated the transcription of VPA1027-1024 and VPA1043-1028. Taken together, these results suggest that CpsQ may directly repress T6SS2 expression in *V. parahaemolyticus*.

In conclusion, our findings showed that CpsQ regulated the transcription of 567 genes, including polar flagella, T6SS2, c-di-GMP metabolism-associated, putative regulatory, antioxidative, and outer membrane protein-encoding genes. CpsQ might directly repress the T6SS2-associated operons VPA1027-1024 and VPA1043-1028. Collectively, this study confirmed the new regulatory roles of CpsQ in *V. parahaemolyticus*.

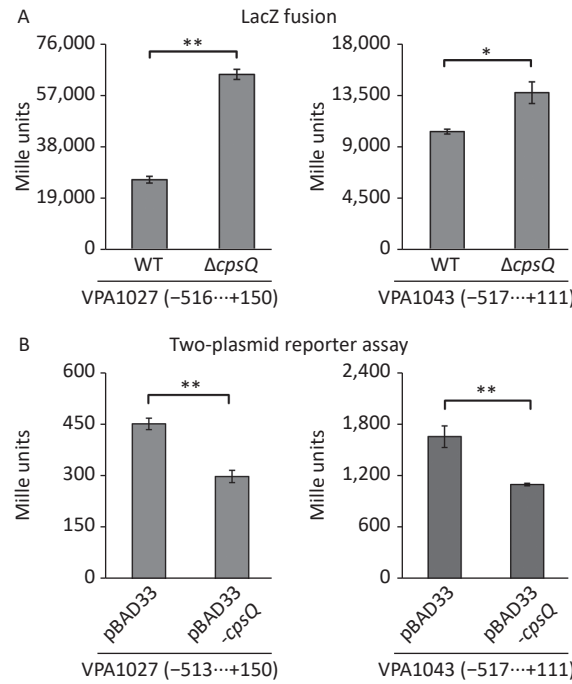

Figure 3. The negative and positive numbers indicate the nucleotide positions upstream and downstream of indicated genes, respectively. Paired Student's *t*-test was used to calculate statistical significance, with a *P*-value less than 0.05 indicating significant differences. $^{**}P < 0.01$; $^{*}P < 0.05$. (A) LacZ fusion. The regulatory DNA region of each target gene was cloned into the pHRP309 plasmid and then transferred into *ΔcpsQ* and WT to determine the promoter activities in cellular extracts. (B) Two-plasmid reporter assay. *E. coli* 100 λpir (Epicentre) bearing pBAD33 or pBAD33-*cpsR* and a recombinant *lacZ* plasmid were grown in LB broth containing 0.1% L-arabinose to mid-log phase, and then aliquots were collected and assayed for *lacZ* expression using the β-galactosidase assay.

Data Availability The original data presented in this study are included in the article and supplementary materials. Further inquiries can be directed to the corresponding authors. Raw RNA-seq data were deposited in the NCBI repository under the accession number PRJNA914418.

Acknowledgements This study was supported by the Natural Science Research Project of the Nantong Science and Technology Bureau (Grant No. JC2021027) and Research Project of the Nantong Health Commission (QN2022044).

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Received: December 12, 2023;

Accepted: February 23, 2024

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