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A novel Diguanylate cyclase VdcR has multifaceted regulatory functions in the pathogenicity of *Vibrio vulnificus*

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ABSTRACT

Background: Vibrio vulnificus is a Gram-negative pathogen that infects humans through foodborne or wound infections. Victims of *V. vulnificus* infections face significant health risks, including cellulitis and septicemia, which have rapid disease progression and high mortality rates. Diguanylate cyclase is responsible for producing the secondary messenger cyclic di-GMP. It plays a crucial role in regulating various bacterial physiological processes, such as motility, toxicity, and pathogenicity, through transcriptional regulation and affecting cyclic di-GMP levels. However, the DGC-mediated pathogenicity regulation in *V. vulnificus* is still unclear.
Methods: The *vdcR* gene in *V. vulnificus* was studied using a deletion strain (ΔVdcR) and an overexpression strain (oeVdcR) to understand its role in regulating the bacterium's pathogenicity. The electrophoretic mobility shift assay and RT-qPCR confirmed VdcR's impact on phosphodiesterase gene expression. To investigate how VdcR affects pathogenicity, *V. vulnificus* variant strains were assays for hemolysis, metalloprotease activity, cytotoxicity, resistance to phagocytosis, and lethality assays of the nematode *Caenorhabditis elegans* after infection.
Results: This study discovered a virulence-associated diguanylate cyclase, VdcR, which serves as a transcriptional regulator to induce phosphodiesterases and reduce the accumulation of cyclic di-GMP. VdcR expression resulted in low hemolysis, metalloprotease, and cytotoxicity activity. It also improved the cell adhesion ability and anti-phagocytosis activity to infect the host cell and escape the macrophage phagocytosis. The constitutively expressed VdcR in *V. vulnificus* caused low mortality rates in *Caenorhabditis elegans* survival assays.
Conclusion: The above evidence demonstrated that VdcR suppresses the pathogenicity in *V. vulnificus* YJ016.

1. Introduction

Vibrio vulnificus is a harmful bacterium commonly found in warm, brackish water ecosystems. It exists both independently and in association with plankton and fish. In its free-living form, it can be ingested by filter-feeding organisms like oysters and temporarily accumulate within them. Eating raw or undercooked seafood that has been contaminated with *V. vulnificus* can cause symptoms such as vomiting, diarrhea, and stomach pain. The free-living form of *V. vulnificus* can contact and adhere to an open wound, colonize it, and then take advantage of the breakdown of the epithelial barrier to invade and cause cellulitis, a rapidly spreading skin infection, and septicemia, which can be fatal. The mortality rate of hospitalized patients who are seriously infected by *V. vulnificus* is high, exceeding 50 % on average.[1–3] Antibiotic treatment is the primary therapeutic regimen for *V. vulnificus* infection. *V. vulnificus* frequently exchanges DNA by horizontal gene transfer that generates its heterogeneity and has been classified into three biotypes.[4] Biotypes 1 and 3 are composed of human clinical strains, while Biotype 2 contains eel pathogens. Analysis of *V. vulnificus* isolates using multilocus sequence analysis revealed that these strains were categorized into phylogenetic lineages 1 and 2, which were predicted by the nucleotide sequence of the virulence-correlated gene.[5] The multifunctional auto-processing RTX toxin is one of the major virulence factors in *V. vulnificus*. It can be divided into subgroups based on its effector domain region analysis.[6]

There are several virulence factors of *V. vulnificus* have been

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reported, including hemolysin pore-forming toxin protein VvhA,[7,8] multifunctional auto-processing RTX toxin (MARTX),[8–11] capsular polysaccharide (CPS), and exopolysaccharide (EPS).[12–15] Since *Vibrio vulnificus* thrives in marine environments and its symbiotic hosts include fish and shellfish, it must employ various strategies to adapt to environmental stresses.[16,17] Cyclic dinucleotides are recognized as critical signaling molecules that regulate various biological processes in bacteria.[18] Cyclic di-GMP (c-di-GMP) is the best-studied cyclic dinucleotide that regulates biofilm production, colony morphology, motility, virulence factor production, and CPS/EPS production.[18–21] Secondary messenger c-di-GMP was synthesized by diguanylate cyclase (DGC) and degraded by phosphodiesterase (PDE). Diguanylate cyclase typically contains a conserved GGDEF domain and is often associated with regulatory domains such as PAS, HAMP, or cyclic mono-nucleotide binding domain (GAF).[22–25] According to the differential construction of the regulatory motif and its receiver domain, DGC-PDE systems could regulate many cellular processes by modulating intracellular accumulated c-di-GMP. It has been reported that some DGC-PDE systems were also regulated by two-component systems to promptly respond to differential environmental stresses.

In *V. vulnificus*, diguanylate cyclase protein A, DcpA, was discovered as a c-di-GMP producer to regulate biofilm formation, rugose colony formation, motility, and virulence.[26] The c-di-GMP regulation on transcription of brp locus (biofilm and rugose polysaccharide), which contains nine genes (brpABCDFHIJK) in *V. vulnificus*, had been discovered to modulate biofilm production.[26,27] Amino acid sequences of BrpR and BrpT of *V. vulnificus* shared 79 % and 33 % of sequence identity to transcriptional regulators VpsR and VpsT of *V. cholerae*, respectively.[28] Both VpsR and VpsT are regulated by c-di-GMP binding to activate the transcription of the *Vibrio* polysaccharide synthesis (*vps*) locus, *vpsI*, and *vpsII*.[29] BrpR and intracellular accumulated c-di-GMP regulate the gene expression of BrpT.[30] The specific binding of BrpT to brpA and brpH promoter sequences, respectively, showed the critical transcriptional regulation to the *brp* locus.[30] This BrpT-mediated *brp* locus gene regulation modulates biofilm production and rugose colony morphology.[31]

The virulence factors VvhA and VvpE are induced by cAMP response protein (CRP) by sensing nutrient availability.[32–36] The transcriptional regulator SmcR regulates virulence genes by sensing increased cell density.[35] IscR and HlyU also regulate virulence genes in *V. vulnificus*.[32,33] In *V. cholerae*, CdgC is a GGDEF-EAL domain-containing protein that regulates rugose colony development, biofilm formation, motility, and virulence.[37] The repression transcription of *aphA*, *tcpP*, *ctxA*, and *tcpA* by CdgC resulted in the down-regulation of *V. cholerae* virulence gene expression.[37] It suggests that GGDEF-containing proteins can regulate downstream response regulators through c-di-GMP accumulation, and some can regulate through transcriptional regulation. Several open reading frames are annotated as GGDEF and/or EAL domain-containing proteins in *V. vulnificus,* while their downstream regulation is still unclear. This study uncovered a virulence-associated diguanylate cyclase, VdcR, with a transcriptional regulator activity that induced PDEs expression to digest cyclic di-GMP and suppressed the pathogenicity in *V. vulnificus*.

2. Methods

2.1. V. vulnificus variants construction and culture condition

The detailed procedures for constructing *V. vulnificus* variants are shown in the Supplemental materials. The nucleotide sequence of VdcR (VVA0351) was obtained from the UniProt database. The upstream and downstream 500 bp regions of VdcR were constructed into a pDS132 vector to create the VdcR gene knockout strain (ΔVdcR) using homologous recombination. The resulting constructs were then transformed into *E. coli* S17 as the DNA donor, while *V. vulnificus* was prepared as the DNA recipient for DNA conjugation. The resulting *vdcR* knockout strain was confirmed by PCR. The VdcR coding gene was inserted into a pET28a vector with a 6-His-tag fusion at both ends. The constructed plasmid was transformed into *V. vulnificus* WT or strain ΔVdcR by electroporation, resulting in VdcR overexpression strain (oeVdcR) or functional complement strain (cVdcR), respectively. *V. vulnificus* variants are routinely cultured in Luria-Bertani (LB) medium (BD Bacto™) with appropriate antibiotic at 37 °C with shaking for 12–16 h. VdcR expression was induced by 1.0 mM of IPTG in strains oeVdcR and cVdcR.

2.2. RNA preparation and the determination of gene expression

The detailed procedures were provided in Supplemental Materials. Briefly, to determine the gene expression of target genes among *V. vulnificus* variants, the cultures were harvested by centrifugation and lysed with NC RNA extraction reagent (EBL Biotechnology, MRE-N3200). The RNA was then isolated, treated to remove genomic DNA, and used to create cDNA for qPCR analysis. The gene expression of bacterial 16s rRNA was used as the control. All the reverse transcription primer pairs were listed in the S1 Table. The 2^- ΔΔCt value was calculated using ΔCt of the target compared to WT, which means the relative gene expression level. All the data was calculated and averaged from at least biological triplicate experiments.

2.3. Motility, hemolysis, and metalloprotease assays

The *V. vulnificus* variants from overnight cultures were transferred to a fresh LB medium with antibiotics and IPTG. The log-phase cultures were harvested and quantified to OD_{600} 0.01. Two microliters of each sample were spotted on LB agar plates with 0.3 % agar and incubated at 37 °C for 24 h to observe bacterial motility. After incubation, the diameter of each swimming zone was determined and averaged from triplicate experiments using Image J. The relative motility was calculated based on the diameter of the WT swimming zone, which was defined as 100 %.

Both hemolysis and metalloprotease assays were carried out on plates to observe the clear zones, and the activities were quantified based on the incubation of the liquid reaction mixture. The hemolysis activity of *V. vulnificus* variants was observed by incubating the cultures on LB agar plates with 5 % sheep-defibrinated blood. After 24 h at 37 °C, a hemolytic clear zone was observed. To quantify the hemolysis activity, the cultures were incubated in the LB medium with sheep-defibrinated blood to detect time-dependent hemolysis activity. The reaction without bacterial cells was defined as negative control (NC), while the reaction containing Radio Immunoprecipitation Assay (RIPA) lysis buffer (Visual Protein, RP05-100) was defined as positive control (PC). Hemolysis activity after broth incubation was calculated using the equation [(sample – NC)/(PC-NC)] x 100. All data were averaged from at least three biological triplicate experiments.

The metalloprotease activity of *V. vulnificus* variants was tested by spotting quantified cultures on LB agar plates containing 3 % skimmed milk and incubating them at 37 °C for 24 h. To quantify the activity, extracellular metalloprotease was harvested from overnight cultures, and a colorimetric assay was performed using azocasein. The reaction mixture contained 200 μL of samples and 200 μL of azocasein (Sigma, 5 mg/mL) that was incubated at 37 °C and terminated by adding 700 μL of 5 % TCA. After centrifugation, 100 μL of supernatant of each sample was mixed with 100 μL of NaOH (0.5 N) for colorization. The A440 was determined to quantify the relative metalloprotease activity among *V. vulnificus* variants.

2.4. Colony morphology and polysaccharide matrix determination

To observe colony morphology, *V. vulnificus* variants were spotted on LB agar plates containing Congo red and incubated for 48 h at 37 °C. The extracellular polysaccharide matrix, or EPS, was quantified using Congo red dye, described in detail in Supplemental Materials. The relative EPS production was calculated using the equation: relative EPS production

= [Blank Control (BC)-sample]/[BC-WT]x100. All data was averaged from at least three biological triplicate experiments.

2.5. Cell adhesion, cytotoxicity, and phagocytosis assays

Colorectal epithelial cells (SW480) are used for cell adhesion and cytotoxicity assays to simulate foodborne infection. The murine macrophage J774A.1 cells are utilized to determine the anti-phagocytosis assays. The SW480 cells are incubated in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco) with 10 % Fetal Bovine Serum (FBS, Gibco) and 1x antibiotic solution (HIMEDIA, A001A-100 ML) which contained 100 U Penicillin and 0.1 mg Streptomycin per mL. The J774A.1 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10 % FBS. The Supplemental Materials describe the detailed procedures for cell adhesion, cytotoxicity, and phagocytosis assays.

2.6. The lethality assays of C. elegans

The *C. elegans* lethality assays followed established procedures.[38] Worms were raised on Nematode Growth Medium (NGM) agar plates seeded with *E. coli* OP50 at 20 °C. For the assays, worms were transferred to a Heart Infusion (HI) agar medium (BD Bacto™) seeded with *E. coli* OP50 as the negative control or seeded with *V. vulnificus* variants for lethality assays. The survival worms were recorded daily during the two-week incubation at 20 °C. Each data point represents the mean ± SD from biological triplicate experiments with n ≥ 120.

2.7. Statistical analysis

The Dunnett's multiple comparisons test was performed to compare WT and each of the other *V. vulnificus* variants. A P value less than 0.05 was defined as statistically significant. GraphPad Prism 7 was applied for statistical analysis.

3. Results

3.1. The construction of VdcR derivative V. vulnificus variants

According to the UniProt database, *V. vulnificus* YJ016 has 46 GGDEF-only and 20 GGDEF-EAL domain-containing proteins. The GGDEF domain-containing protein VdcR (VVA0351) has 527 amino acids and a theoretical molecular weight of 59792.7 Da. The 3D structure of VdcR was predicted by Alphafold (Fig. 1A). Residues K408, N411, D412, H416, D420, R443, G445, and E448 are predicted active sites, while the highly conserved GGEEF active site is located at residues 445–449. The coding gene was constructed into a pET28a vector and transformed into *V. vulnificus* YJ016 as a VdcR overexpression strain (oeVdcR). The VdcR deletion strain (ΔVdcR) and the functional recombinant strain (cVdcR) were also constructed for the study.

3.2. Constitutive expressed VdcR induces phosphodiesterase expression

The gene expression of VdcR in the *V. vulnificus* variants was confirmed using real-time qPCR, which primer sequences showed in S1 Table. Both the oeVdcR and cVdcR strains showed significantly higher levels of *vdcR* gene expression (Fig. 1B). The TPR-like domain in VdcR may participate as a response regulator to drive protein-protein interaction. Sequence analysis by DNABIND showed that VdcR has a 70 % probability of DNA binding ability, while Alphafold predicted the 3D structure of VdcR for DNA binding prediction showed a 95 % DNA binding ability (S2 Table). To validate the DNA binding ability of VdcR,

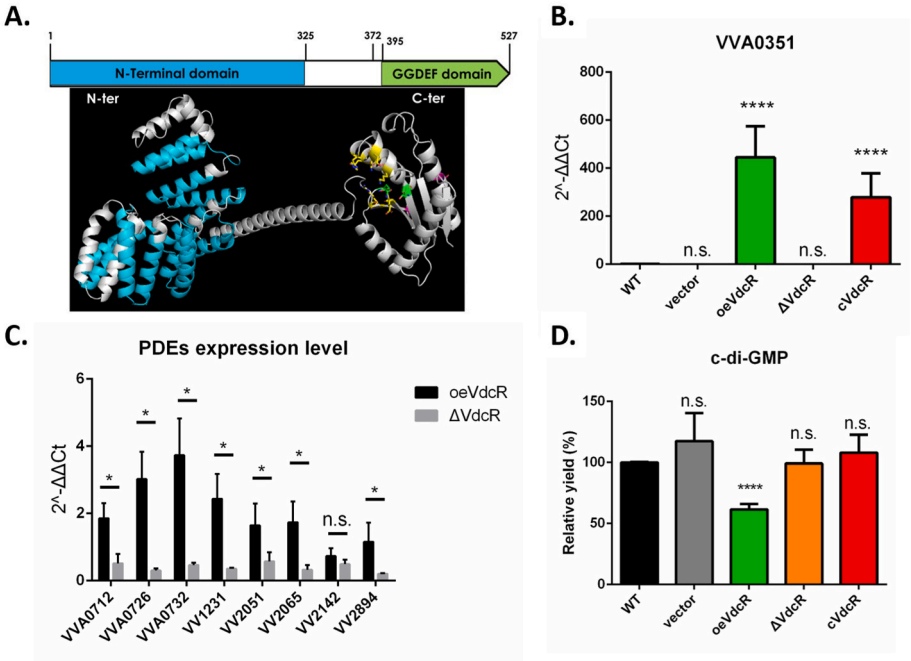

Fig. 1. The expression of VdcR induces gene expression of PDEs in *V. vulnificus*. (A) The annotated tertiary structure of VdcR. The tertiary structure of VdcR was predicted by Alphafold and displayed by PyMol. The N-terminal putative TPR-like domain is marked in cyan. The C-terminal GGDEF domain (395–527) has active sites (yellow), metal ion-binding sites (green), and product inhibition sites (I-sites, purple). **(B) The VdcR gene expression levels among *V. vulnificus* variants.** The value of 2^-ΔΔCt indicated the gene expression level of *vdcR* compared to WT, which are both normalized with the expression level of 16s rRNA. Data were averaged from biological triplicate experiments. **(C) The gene expression of PDE was induced by VdcR.** The RT-qPCR was performed in at least three biological repeats and averaged from triplicate data. The 2^-ΔΔCt values indicated that each PDE's gene expression levels were normalized to 16s rRNA, with gene expression relative to WT. **(D) Cyclic di-GMP production among *V. vulnificus* variants.** The accumulated c-di-GMP of each *V. vulnificus* variant was determined by Cyclic-di-GMP Assay Kit (BioVision). Data are presented as mean ± SD, n ≥ 3. The ANOVA analysis generated the P value to show the difference between WT and each of the other *V. vulnificus* variants. n.s., not significant at $P > 0.05$ (*$P < 0.05$ and ****$P < 0.0001$).

the N-terminal TPR-like domain was overexpressed and purified for Electrophoretic Mobility Shift Assay (EMSA), as described in Supplemental Materials, confirming its DNA binding ability (S1 Fig). To figure out whether VdcR regulates expression of PDE in *V. vulnificus*, we select 8 PDEs as targets, including 4 EAL domain-containing proteins (VVA0712, VVA0732, VV1231, and VV2142) and 4 HD-GYP domain-containing proteins (VVA0726, VV2051, VV2065, and VV2894) for gene expression analysis by using primers listed in the S1 Table. Results reveal that the gene expression of eight PDEs was induced when *vdcR* was overexpressed in strain oeVdcR (Fig. 1C). In contrast, the expression of these PDEs was suppressed when *vdcR* was knockout in strain ΔVdcR that demonstrated VdcR have the transcriptional regulation function to induce the transcription of PDEs. The accumulated cyclic-di-GMP in the wild-type strain was 71.34 nM. In contrast, strain oeVdcR showed the lowest level of accumulated cyclic di-GMP among *V. vulnificus* variants (Fig. 1D), indicating VdcR's role in inducing phosphodiesterase expression.

3.3. VdcR modulates the motility, colony morphology, and EPS production in V. vulnificus

The motility of *V. vulnificus* variants was tested by incubating them on agar-containing LB plates. VdcR significantly inhibited motility, reducing relative motility in certain strains (Fig. 2A and S2). VdcR also influenced colony morphology (S3 Fig) and EPS production (Fig. 2B), with strain oeVdcR producing significantly higher EPS than WT, while strain cVdcR showed a moderate increase. These findings demonstrate the regulatory role of VdcR in motility and EPS production in *V. vulnificus*.

3.4. VdcR suppresses the hemolysis and casein digestive activity in V. vulnificus

The hemolysis and metalloprotease activities are key virulence factors of *V. vulnificus*. The expression of VVH (*vvhA*) and VVP (*vvpE*) is regulated by bacterial quorum sensing systems. Gene expression levels of *vvhA* and *vvpE* were slightly induced by VdcR but not significantly (Fig. 3A and B), indicating that VdcR does not regulate VVH and VVP at the transcriptional level.

The study investigated hemolysis and metalloprotease activity regulation in different *V. vulnificus* variants. Results showed that the oeVdcR variant had the lowest hemolysis activity, while the WT variant exhibited significant hemolysis ability (Fig. 3C and S4). The cVdcR strain significantly suppressed hemolysis activity within 1 h of incubation, while oeVdcR suppresses hemolytic activity in *V. vulnificus* for up to 24 h.

The metalloprotease activity of *V. vulnificus* variants was observed using LB agar plates containing 3 % skimmed milk. After 48 h of incubation, the WT, ΔVdcR, and cVdcR strains showed a hydrolysis ring, indicating their metalloprotease activity, while the strain oeVdcR did not (S5 Fig). The quantified protease activity showed that strain oeVdcR had only 15.4 % of the metalloprotease activity of the WT (Fig. 3D). The protease activity of oeVdcR was significantly suppressed. At the same time, it was improved considerably in the ΔVdcR strain. This result demonstrates that the metalloprotease activity of *V. vulnificus* was suppressed by VdcR.

3.5. Cell adhesion activity of V. vulnificus was improved by VdcR

The initial step of bacterial infection is adhesion to the host cell. To mimic the foodborne infection of *V. vulnificus*, the colorectal epithelial cell SW480 was utilized to determine the cell adhesion activity. The cell adhesion activity of *V. vulnificus* variants was determined at a MOI of 10 for 1 h incubation. The findings indicate that the WT exhibited low cell adhesion activity since the attached colonies were less than 100 (Fig. 4A). In contrast, oeVdcR had more than 10 to the power of 6 cells attached to SW480 (Fig. 4A). This result demonstrated that the constitutively expressed VdcR significantly promoted the cell adhesion activity in *V. vulnificus*.

3.6. The cytotoxicity and anti-phagocytosis activity of V. vulnificus was regulated by VdcR

The lethal effects of *V. vulnificus* on mice have been proven to be primarily caused by the potent RtxA1 toxin, which induces cytoskeletal rearrangement and acute cytotoxicity.[39,40] To investigate whether VdcR regulates *V. vulnificus*' cytotoxicity during foodborne infection, the colorectal epithelial cell SW480 was utilized to determine the cytotoxicity activity. The cytotoxicity of VdcR-mediated *V. vulnificus* variants was measured by colony formation assays at a MOI of 10 for 2 h incubation. The relative cell viability of SW480 when treated with oeVdcR and cVdcR was significantly higher than WT (Fig. 4B). It reveals the *V. vulnificus*' cytotoxicity was suppressed considerably by VdcR.

The MARTX cytotoxin in *V. vulnificus* can inactivate the Akt-mediated signaling pathway within macrophages, impairing the macrophage's ability to phagocytize the infected bacteria.[41] The number of viable colonies of WT after being phagocytosed by macrophages is less than 10, whereas the number of colonies within J774A.1 infected with

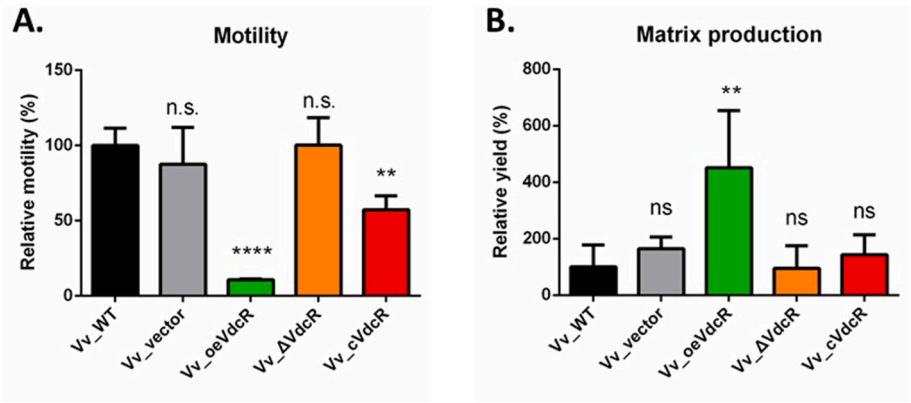

Fig. 2. The bacterial motility and EPS production of *V. vulnificus* were suppressed by VdcR. (A) Motility assays of *V. vulnificus* variants. The overnight cultures of *V. vulnificus* variants were diluted to OD_{600} 0.01 by LB medium for spotting 2 μL on 0.3 % agar containing LB plate. The motility of *V. vulnificus* variants was determined according to their swimming zone. The relative motility was calculated from at least biological triplicate experiments. **(B) EPS determination of *V. vulnificus* variants.** The *V. vulnificus* variants were cultured on LB agar plates containing 50 μg/mL Congo red for 48 h incubation at 37 °C. The amount of EPS was determined and calculated from biological triplicate experiments. Data are presented as mean ± SD, n ≥ 3. The ANOVA analysis generated the P value to determine the significant difference between WT and each of the other *V. vulnificus* variants. n.s., not significant at $P > 0.05$ (**P < 0.01 and ****P < 0.0001).

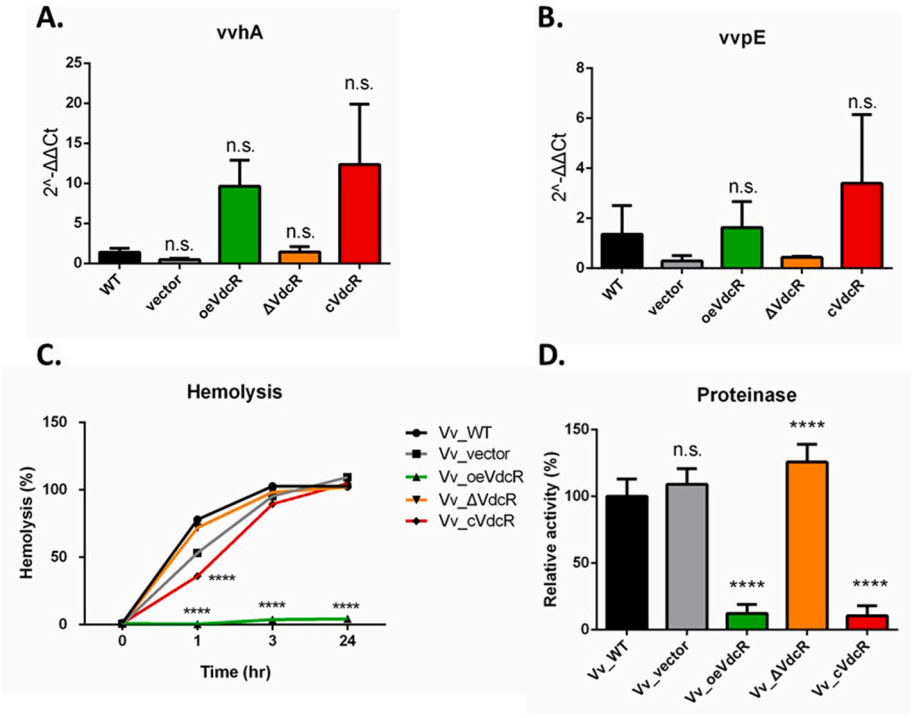

Fig. 3. Gene expression of (A) *vvhA* and (B) *vvpE* in *V. vulnificus* variants. The RT-qPCR was performed in at least three biological repeats and averaged from triplicate data. **(C) Hemolysis activity assays of *V. vulnificus* variants.** The relative hemolysis activity was determined and calculated from biological triplicate experiments. The values of the lysate of red blood cells treated with RIPA buffer are defined as 100 % as the positive control to calculate the relative hemolysis activity. **(D) Casein digesting activity assays of *V. vulnificus* variants.** The relative azocasein digesting activity was quantified from biological triplicate experiments. Data are presented as mean ± SD, n ≥ 3. The ANOVA analysis generated the P value to determine the significant difference between WT and each of the other *V. vulnificus* variants. In panel C, the relative hemolysis activity of oeVdcR and cVdcR was compared to WT at each time point. n.s., not significant at $P > 0.05$, while **** indicated $P < 0.0001$.

oeVdcR reached ten powers of 4. (Fig. 4C). These results demonstrate that VdcR could suppress *V. vulnificus*' cytotoxicity and promote the anti-phagocytosis activity to defend the host's immune system.

CRP, a cAMP response protein, has been reported to regulate cytotoxicity and anti-phagocytosis activities in *V. vulnificus*.[34–36] HlyU is one of the critical transcription factors involved in the pathogenicity of *Vibrio* species to induce both *rtxA* and *vvhA* expression.[42] It is known that SmcR and CRP repress the expression of HlyU. To investigate the relationship between VdcR to *crp* and *rtxA*, the expression of both coding genes was quantified in WT and oeVdcR conditions (Fig. 4D). The result demonstrated that both *crp* and *rtxA* gene expression were significantly induced by VdcR (Fig. 4D). It was revealed that VdcR might be an upstream regulator to modulate *crp*- and *rtxA*-mediated regulation in *V. vulnificus*.

3.7. The lethality assays of C. elegans infected by V. vulnificus variants

The pathogenicity of different *V. vulnificus* variants was tested using *C. elegans* as a host model. The survival rate of nematodes fed on *V. vulnificus* variants differed, with the oeVdcR variant showing suppressed pathogenicity (Fig. 5). For the quantitative analysis of the results, the time required to kill 50 % of adult *C. elegans* was defined as TD50 to display the differential pathogenicity between *V. vulnificus* variants. The nematodes' TD50 values when fed with WT, ΔVdcR, and oeVdcR are 9, 1, and 13 days, respectively. The results indicate that *V. vulnificus* is highly toxic, causing significant lethality in *C. elegans*, while VdcR significantly suppressed its pathogenicity.

4. Discussion

This study utilized the GGDEF domain-containing protein VdcR to investigate the regulation of bacterial motility, EPS production, hemolysis, metalloprotease activity, cell adhesion, cytotoxicity, and anti-phagocytosis activity. The RT-qPCR results confirm the expression of VdcR in *V. vulnificus* variants we constructed in this study. The expression of VdcR may be a critical factor in modulating pathogenicity in *V. vulnificus*. Under regular growth conditions, gene expression of VdcR is very low (Fig. 1B), resulting in the ΔVdcR strain having similar phenotypes with WT. It reveals that VdcR expression may be regulated by specific factors, which require more solid experimental evidence to validate it. Even though the gene expression of VdcR in the cVdcR strain is significantly higher than WT, it is still lower than oeVdcR, which leads to weaker VdcR-mediated effects in the cVdcR strain.

The production of capsular polysaccharide (CPS) is a significant virulence factor in various bacteria. EPS and CPS production regulate biofilm formation in *V. vulnificus*. In this study, the VdcR-mediated regulation in the polysaccharide matrix production is consistent with previous evidence that overexpression of DGC (DcpA) enhances EPS production and results in the rugose colony morphology in *V. vulnificus* strain 27562.[26] The constitutively expressed VdcR suppresses *V. vulnificus*' motility and promotes its polysaccharide matrix, prompting us to hypothesize that it may have better infection ability.

The qPCR results revealed that both *crp* and *rtxA* gene expressions were induced by VdcR, demonstrating that VdcR may modulate *crp*-mediated cytotoxicity and *rtxA*-mediated anti-phagocytosis regulation. In this study, VdcR significantly suppressed cytotoxicity, which is consistent with the phenotype of *V. vulnificus* when CRP is expressed.[34–36] The *rtxA* gene expression was significantly increased by VdcR, leading to higher anti-phagocytosis activity. The VdcR-mediated induction of RtxA1 did not result in a higher cytotoxicity phenotype because the other critical exotoxins, VvhA and VvpE, were significantly suppressed. Furthermore, the effects of VdcR on TolC-mediated RtxA1

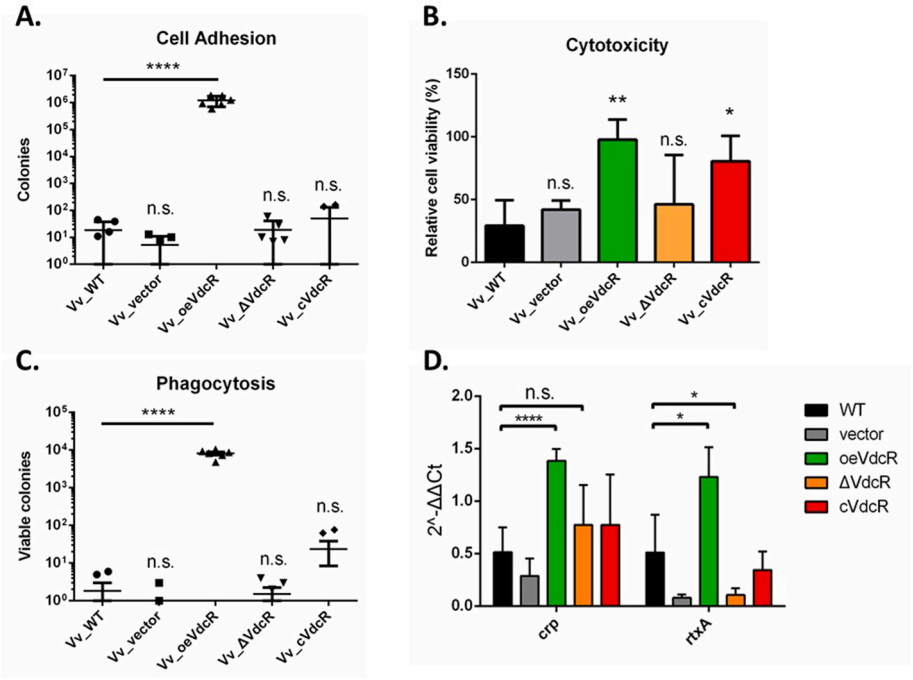

Fig. 4. Pathogenicity assays of *V. vulnificus* variants. (A) Cell adhesion, (B) Cytotoxicity, and (C) Anti-phagocytosis assays of *V. vulnificus* variants. These assays utilized the colorectal epithelial cell SW480 as the mammalian host cells for (A) and (B) while using murine macrophage J774A.1 for (C). The multiplicity of infection (MOI) was 10. The Y-axis of panels A and C indicated the colony number that adhered to SW480 (A) and the colony numbers that were still alive after phagocytosis by J774A.1 (C). Data are presented as mean ± SD, n ≥ 3. The ANOVA analysis generated the P value of WT against each of the other *V. vulnificus* variants. n.s., not significant at $P > 0.05$ (*P < 0.05, **P < 0.01, and ****P < 0.0001). (D) **Gene expression of *crp* and *rtxA* among *V. vulnificus* variants.** Data are presented as mean ± SD, n ≥ 3. The ANOVA analysis generated the P value of WT against oeVdcR or ΔVdcR, respectively. n.s., not significant at $P > 0.05$ (*P < 0.05, and ****P < 0.0001).

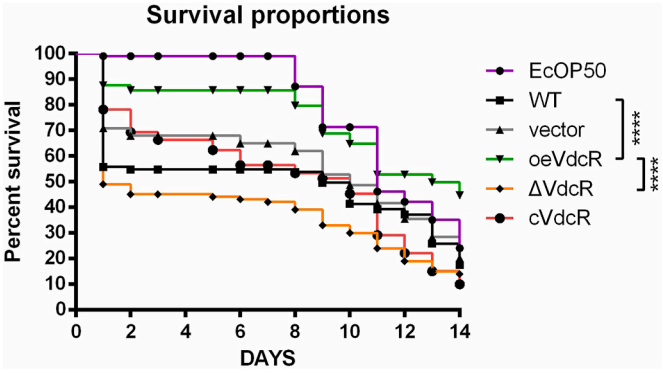

Fig. 5. The pathogenicity of *V. vulnificus* was suppressed by VdcR. *C. elegans* was utilized as a host model to determine the survival percentage when fed with *V. vulnificus* variants in this study. Data are presented as mean from biological triplicate experiments, n ≥ 120. *P* value was generated by the log-rank test to show the significant difference between groups with **** indicating the P < 0.0001.

secreting protein are still unknown. It might be one reason why VdcR induce the *rtxA1* gene expression but diminished *V. vulnificus*' cytotoxic activity. These results suggest that VdcR is involved in *V. vulnificus*' pathogenic modulation, while the detailed regulatory pathway requires more experimental evidence for validation.

In conclusion, VdcR is a transcriptional regulator in *V. vulnificus* that influences various pathogenic activities. It promotes anti-phagocytosis activity and polysaccharide matrix production while suppressing motility and cytotoxicity. This makes VdcR a potential target for developing new therapeutic interventions against *V. vulnificus*.

CRediT authorship contribution statement

Yi-Wen Chen: Writing – original draft, Investigation, Formal analysis, Data curation. **Tien-Sheng Tseng:** Writing – review & editing, Validation, Investigation, Data curation. **Kai-Ting Chen:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Shu-Jung Lai:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data statement

The authors confirm that the data supporting this study's findings are available within the article and its supplementary materials.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.11.013.

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