OpA and RpoS are positive regulators of a virulence factor PrtA in Vibrio parahaemolyticus

San-Chi Chang and Chia-Yin Lee*

Abstract

PrtA is an extracellular serine protease of Vibrio parahaemolyticus and has haemolytic and cytotoxic activities. Many extracellular proteases have been shown to be required for nutrient intake and the infection mechanism of vibrios. In this study, we report that OpA, a quorum sensing regulator, and RpoS, a general stress response regulator, play important roles in the PtA regulation pathway. Extracellular protease activity was highest during the late-log growth of Vibrio parahaemolyticus no. 93 (VP93). The absence of PrtA distinctly decreased the extracellular protease activity. Deletion of opaR or rpoS alone reduced PrtA-specific activity of VP93. Quantitative reverse-transcriptase PCR and Western blot analysis suggested that OpA and RpoS promote PrtA expression at the transcriptional level and affect the amount of extracellular PrtA. A luciferase assay revealed that OpA regulates prtA on the prtA promoter region. Electrophoretic mobility shift assays indicated that the purified His-OpA was able to bind specifically to two sequences (PrtA-1 and PrtA-2) of the prtA promoter region. Footprinting analysis showed that OpA regulates prtA by binding to the promoter region of prtA at positions −269 to −246 and −88 to −68 from the prtA translational start site. Together, the results suggest that PrtA was upregulated by two global regulators, OpA and RpoS.

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium that was discovered in Japan in 1950 during a food poisoning outbreak [1]. It lives within marine and estuarine environments and can be found in multiple cell types, including free-swimming forms and colony formations attached to inert surfaces of organisms under water, such as shellfish [2]. When humans are infected with V. parahaemolyticus via consumption of raw contaminated seafood, they develop acute gastroenteritis and inflammation [3]. Many virulence factors of V. parahaemolyticus are known, including thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH), lethal toxins, ureases, adhesion factors, extracellular proteases and type III secretion system (T3SS) effectors [4].

The well-known haemagglutinin/protease (HAP) of Vibrio cholerae plays an important role in bacterial virulence [5–9]. Some extracellular protease is involved in bacterial autolysis [10]. The wild-type strain used in this study is V. parahaemolyticus no. 93 (VP93) isolated from an outbreak of food poisoning in Taiwan. As reported previously, VP93 is a tdh− and trh− strain. To identify the virulence factor of VP93, we separated each extracellular protein based on molecular weight and examined all protease activities. Among them, we found that one protease is a metalloprotease [11] while another possessing the highest protease activity is an alkaline serine protease, PrtA. PrtA is toxic to various mammal cells and causes haemolysis of erythrocytes. The LD50 of purified PrtA and VP93 bacteria ranged from 2.5 to 5 mg kg−1 in mice via the intraperitoneal route and 1 × 1011 c.f.u. via the oral route [12].

Quorum sensing (QS) is a signal transduction system for bacteria to communicate with each other using extracellular chemicals [13]. These extracellular chemicals are called autoinducers. In QS system, bacteria detect the amount of autoinducers to determine the cell population density and synchronize the gene expression of the group [14]. QS pathways in members of Vibrionaceae are similar. Major QS regulators in the family Vibrionaceae are conserved [15]. The QS regulator in V. parahaemolyticus is OpA, a homologue of LuxR [16] in V. harveyi. OpA was discovered in 1998...
and found to be able to change the colony morphology of *V. parahaemolyticus* [17]. In recent studies, researchers have integrated the binding sequences of the major QS regulators in *Vibrioaceae*, including LuxR in *V. harveyi* (ATCC BAA-1116), OpaR in *V. parahaemolyticus* (RIMD 2210633), SmcR in *V. vulnificus* (YJ016) and ValR in *V. alginolyticus* (ZJ-51). The proposed binding sequence of the major QS regulator was TATTGATATA-TTTAT-CAATA and named master quorum sensing regulator (MQSR) box [18]. In bacteria, several sigma factors control expression of numerous genes under various conditions. RpoS, also referred to as σ^s^ or σ^[^S^]_[^S^], is a subunit of the RNA polymerase holoenzyme. It is a regulator that controls the expression of stress and stationary phase-inducible genes [19, 20]. RpoS also affects the expression of many virulence genes in pathogenic enteric bacteria by distinguishing the promoter sequence [21, 22]. RpoS has an antagonistic effect on the nitrogen limitation sigma factor, RpoN [23].

In this study, we clarify the roles of OpaR and RpoS in the regulation pathway of PrtA in VP93. We show that OpaR enhances prtA expression by binding to the prtA promoter. Experimental analysis revealed that prtA was regulated by RpoS during the stationary phase growth. Both ΔopaR and ΔrpoS decrease the amount of extracellular PrtA and PrtA protease activity in VP93. This is the first report to demonstrate the correlation between PrtA, an extracellular protease virulence factor and global regulators in *V. parahaemolyticus*.

**METHODS**

**Bacterial strains, plasmids and growth conditions**

All bacterial strains, plasmids and primers reported in this study are listed in Tables 1 and S1 (available in the online version of this article). *V. parahaemolyticus* strains were grown in tryptic soy broth containing 3% NaCl (TSB3) at 35°C. *Escherichia coli* strains XL1-Blue [24] and S17-1 λ-pir [25] were grown in Luria–Bertini (LB) (Difco) medium at 37°C. Mutant and reporter strains were all derived from VP93. When necessary, we added antibiotics to the medium at the following concentrations: ampicillin, 100 µg ml^−1^; kanamycin, 25 µg ml^−1^; and chloramphenicol, 20 µg ml^−1^ for *E. coli*, and 5 µg ml^−1^ for *V. parahaemolyticus*.

**Construction of mutants**

Deletion mutants in this study were constructed by homologous recombination. We used the primer sets VP2516D-A2/VP2516D-B1 and VP2516D-C1/VP2516D-D1 to amplify the 5′ and 3′ regions of the *opaR* gene respectively (Table S1). The two regions were annealed via an overlapping PCR to delete 615 bp of *opaR*. This fragment was named *mopaR* and cloned into a suicide vector, pDS132 [26], with SpII and XbaI restriction sites, to yield the pDS132-*mopaR* plasmid (Table 1). This mobilizable plasmid was transferred from *E. coli* S17-1 λ-pir to VP93 by conjugation [27]. The conjugants were selected from a TSB3 Cm5 plate for the first step of the homologous recombination.

Subsequently, TCBS plates containing 6% sucrose were used to select the second homologous recombination conjugants. The *opaR* deletion mutant was confirmed by PCR amplification and sequencing. Other mutants, ΔprtA, ΔrpoS and ΔopaRΔrpoS, in this study were constructed and confirmed in the same way.

**His6-OpaR protein expression and purification**

The *opaR* gene was amplified using primers VP2516-EXF and VP2516-EXR (Table S1) and then cloned into the six-His-tag expression vector, pET28a (Novagen), to generate pET28a-*opaR* plasmid (Table 1). The clone was transformed into *E. coli* BL21 (DE3) to express OpaR with an N-terminal fusion tag. *E. coli* BL21 (DE3) with pET28a- *opaR* was grown in 200 ml LB broth supplemented with 25 µg kanamycin ml^−1^ at 37°C. When the optical density at 600 nm (OD_600) reached 0.4–0.6, 1 mM IPTG was added to induce His6-OpaR expression at 30°C for 4 h. After induction, the cells were collected and disrupted using the Constant Cell Disrupter System (Constant SystemK). We purified His-OpaR using His GraviTrap (GE Healthcare) and condensed it with Amicon Ultra-15 centrifugal filter devices (Merck Millipore). The purity of His6-OpaR was confirmed by SDS-PAGE analysis (Fig. S1). Proteins were quantified using the method described by Bradford [28].

**Quantitative reverse-transcriptase PCR (qRT-PCR)**

We mixed bacterial cultures of *V. parahaemolyticus* with RNA protect bacterial reagent (Qiagen), and collected cells by centrifuging at 5000 g and 4°C for 15 min. Total RNA was isolated from different growth phases of *V. parahaemolyticus* with TRIzol reagent. RT-PCR was run with HiScript I Reverse Transcriptase (Bionovas Biotechnology) according to the instructions of the manufacturer. Real-time qRT-PCR was performed using SYBR Green Master Mix (Applied Biosystems) in a Step One Real-Time PCR System (Applied Biosystems). Relative expression values were determined from 2^−(ΔΔCt Target−ΔΔCt Reference)_. Ct is the fractional threshold cycle and the reference was the 16S rRNA gene. The specific primer sets used in qRT-PCR were: VP2516_279_F and VP2516_531_R for *opaR* mRNA, VP2553_441_F and VP2553_693_R for *rpoS* mRNA, VPA0227_728_F and VPA0227_992_R for *prtA* mRNA, VPA0226_346_F and VPA0226_601_R for *ldh* mRNA, and VPAr01_132951_F and VPAr01_133200_R for 16S rRNA (Table S1).

**prtA-luxAB promoter fusion and luciferase assay**

We used primer set VPA0227_LuxAB_F and VPA0227_LuxAB_R (Table S1) to amplify the 480 bp fragment of the *prtA* promoter. This fragment was cloned into a promoterless vector, pSAluxAB (Table 1), with restriction sites SmaI and XbaI to generate pSAluxAB-prtA (Table 1). This plasmid was transformed to VP93 and its mutant strains ΔrpaR, ΔrpoS and ΔopaRΔrpoS using the Gene Pulser Xcell Electroporation System (Bio-Rad). Luciferase activity was measured by adding n-decanol (final concentration 0.001%) as substrate to 2 ml bacterial cultures. Luminescence was detected on a Spectrofluorometry F-2500 device (Hitachi)
**Table 1. Strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
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<tr>
<td><em>V. parahaemolyticus</em> no. 93</td>
<td>tdi' trh*</td>
<td>[11]</td>
</tr>
<tr>
<td>ΔopAR</td>
<td>no. 93 ΔopAR</td>
<td>This study</td>
</tr>
<tr>
<td>ΔrpoS</td>
<td>no. 93 ΔrpoS</td>
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<tr>
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<td>no. 93 ΔopARΔrpoS</td>
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<tr>
<td>ΔprtA</td>
<td>no. 93 ΔprtA</td>
<td>This study</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<td>DH5α</td>
<td>F′ endA1 glnV44 thi− recA1 relA1 gyrA96 deoR supG46 Δ(hisD46 lacZΔM15 lacZYA−argF80)</td>
<td>Invitrogen</td>
</tr>
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<td>BL21(DE3)</td>
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<td>Novagen</td>
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<td>RBC</td>
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<td>[26]</td>
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<td>pET28a</td>
<td>Expression vector, Km′</td>
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<td>pSA19CP vector with luxAB reporter gene, Cm′</td>
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<tr>
<td>pET28a-opaR</td>
<td>pET28a containing opaR coding region, Km′</td>
<td>This study</td>
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* Amp′, ampicillin resistance; Cm′, chloramphenicol resistance; Km′, kanamycin resistance.

and is shown as specific light units (SLU; relative light units per second per ml per OD600 unit).

**Western blot**

Cultures of *V. parahaemolyticus* strains from each bacterial growth phase were collected by centrifuging at 4000 r.p.m. and 4 °C for 20 min. The supernatants were precipitated using 70 % ammonium sulfate and resuspended using 50 mM Tris buffer. After dialysis overnight, extracellular proteins were concentrated using Amicon Ultra-15 centrifugal filter devices (Merck Millipore). Proteins were separated using 12.5 % SDS-PAGE and transferred to polyvinylidene fluoride membranes (Merck Millipore). The membranes were blocked with 5 % skimmed milk and blotted with PrtA-specific antibody as the primary antibody. The secondary antibody was an anti-rabbit IgG horseradish-peroxidase-linked antibody (Cell Signaling Technology). Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Merck Millipore) was added and signals were detected using a chemiluminescence image system (MultiGel-21; Bio Pioneer Tech).

**Transcription start sites of prtA and ldh determined by 5′RACE**

We applied the modified 5′ rapid amplification of cDNA ends (5′RACE) (adaptor-radioactivity-free transcription start site; ARF-TSS) method of Wang [29] to determine *prtA* and *ldh* transcription start sites. Total RNA was isolated from VP93 and reversely transcribed with 5′-end phosphorylated primers VPA0227-SP1 for *prtA* and VPA0226-SP1 for *ldh*. RNA was degraded using 0.2 M NaOH and neutralized with 0.2 M HCl. Then, T4 RNA ligase (New England Biolabs) ligated the 5′-phosphorylated terminal sequence and 3′-end of the transcription start site (TSS) to form a circular cDNA. The circular cDNA was amplified using two inverse primers: VPA0227-SP2F and VPA0227-SP2 for *prtA*, and VPA0226-SP2F and VPA0226-SP2 (Table S1) for *ldh*. The amplified PCR products were cloned into an RBC TA Cloning Vector (RBC Bioscience) and then sequenced.

**Electrophoretic mobility shift assay (EMSA)**

Primer sets VPA0227-EMSAF1/VPA0227-EMSAR1 and VPA0227-EMSAF2/VPA0227-EMSAR2 (Table S1) were used to amplify EMSA probes, PrtA-1 and PrtA-2, respectively, which are the putative OpaR binding sequences of the *prtA* promoter region. The two fragments were labelled with digoxigenin-11-ddUTP at their 3′ ends using the 2nd generation DIG Gel Shift Kit (Roche Applied Sciences). In the binding reaction, 0.4 ng of labelled fragment was incubated with purified His-OpaR protein in 20 µl binding buffer [100 mM Hepes (pH 7.6), 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT, 1 % (w/v) Tween 20, 150 mM KCl] at 25 °C for 30 min. In competition analysis, protein–DNA complex was mixed with 100 ng unlabelled fragment. These samples were separated by 6 % native PAGE and transferred to a positively charged nylon membrane by electroblotting. Finally, immunological treatment and chemiluminescent signal detection was carried out according to instructions for the 2nd generation DIG Gel Shift Kit (Roche Applied Sciences).
DNase I footprinting assay

The probe sequence of the DNase I footprinting assay was located in the \textit{ldh-prtA} intergenic region. The 5' ends of the sense and antisense probes were both labelled with 6-carboxyfluorescein (6-FAM). The primer sets used in this assay were VPA0227-F-6FAM/VPA0227-R and VPA0227-F/VPA0227-R-6FAM (Table S1). This assay was conducted using a method from Zianni [30]. In the binding reaction, 75 ng labelled probe and various amounts of His-OpaR protein were mixed in 20 µl binding buffer (2nd generation DIG Gel Shift Kit) and incubated at 25°C for 30 min. We added 0.015 U DNase I (Thermo Scientific) at 25°C for 1 min to digest DNA–protein complex and stopped this reaction with 2.77 µl stop solution at 65°C for 10 min. An Agencourt AMPure XP PCR Purification Kit (Beckman Coulter) was used to purify the digested DNA fragments. The digested DNA fragments were added to a mixture of 8.5 µl HiDi formamide and 0.5 µl Gene Scan-LIZ600 size ladder (Applied Biosystems). The samples were analysed on a 3730xl DNA analyser (Applied Biosystems) and with Peak scanner software V1.0 (Applied Biosystems).

Protease activity

Protease activity was determined using Azocoll as the substrate and the procedures were performed as described previously [11]. Purified protein was added to a 1 ml Azocoll suspension and incubated at 37°C on a shaker rotating at 150 r.p.m. for 1 h. After incubation, samples were put on ice for 10 min to stop the reaction and then centrifuged at 12000 r.p.m. for 5 min. The supernatant was detected at OD\textsubscript{520} using a spectrophotometer (U-2900; Hitachi). The blank control was 50 mM Tris-HCl. One unit of enzyme activity was defined as the amount of enzyme that caused a 1 unit increase in OD\textsubscript{520} per hour at 37°C.

Statistical analysis

Statistical differences between the three samples were measured using Student's t test with a two-tailed distribution. \(P\) values of <0.01 were considered statistically significant.

RESULTS

PrtA dominates the extracellular protease activity of VP93

PrtA is a novel putative virulence factor. Extracellular protease activity reached the highest level when VP93 grew to the late-log phase. Interestingly, the extracellular protease activity decreased slightly at the late-stationary phase (Fig. 1a). A deletion mutation in the \textit{prtA} gene was obtained in this study via double-crossover homologous recombination, and the extracellular protease activity of \(\Delta\text{prtA}\) decreased about 17-fold compared with the wild-type strain (VP93) after the log phase (Fig. 1b). These results show that the bulk of extracellular protease activity of VP93 was caused by PrtA. PrtA activity was sustained from log-phase to late-stationary phase.

\textit{opaR}, \textit{rpoS} and \textit{prtA} are expressed in different bacterial growth phases

Because PrtA dominates the extracellular protease activity of VP93, we investigated the regulation pathway of PrtA. Initially, we found that \textit{prtA} is expressed from the bacterial log-phase based on real-time qRT-PCR data (Fig. 2a). Therefore, we hypothesized that \textit{prtA} might be regulated by a high cell density regulator, OpaR, and stationary phase regulator, RpoS [31]. Gene expression levels of \textit{opaR} and \textit{rpoS} from log

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**Fig. 1.** Role of PrtA in extracellular protease activity of VP93. (a) Extracellular protease activity of wild-type VP93 was measured according to the time course of bacterial growth. (b) Protease activity of wild-type VP93 and \(\Delta\text{prtA}\) was detected at the log phase (OD\textsubscript{600} of 0.5), late-log phase (OD\textsubscript{600} of 2), stationary phase (2 h after reaching an OD\textsubscript{600} of 2) and late-stationary phase (4 h after reaching an OD\textsubscript{600} of 2). Values are the mean of three independent experiments. Error bars indicate the standard deviation (*\(P<0.01\)).
to late-stationary phases were also determined by real-time qRT-PCR. As shown in Fig. 2, the *opaR* gene was expressed stably starting from the log growth phase (Fig. 2b), and *rpoS* gene expression increased 5-fold at the stationary growth phase compared to the log phase (Fig. 2c). Moreover, we found no significant effects on growth rate for the wild-type strain VP93, or its mutants Δ*opaR*, Δ*rpoS* and Δ*opaRΔrpoS* (Fig. 2d). Therefore, we used log-phase and stationary-phase cultures to study the association between *prtA* and the two global regulators, *OpaR* and *RpoS*.

### OpaR and RpoS positively regulate expression of *prtA*

Microarray results have shown that deletion of *opaR* reduces the expression of surface sensing regulon genes of motility and secretion systems [32]. *PrtA* is the major extracellular protease of VP93, and thus we investigated the effect of *OpaR* and *RpoS* on *prtA* expression. Real-time qRT-PCR was used to quantify the *prtA* gene expression in wild-type strain VP93, Δ*opaR*, Δ*rpoS* and Δ*opaRΔrpoS* at log and stationary growth phases. Real-time qRT-PCR data show that *prtA* gene expression decreased 2.5-fold in Δ*opaR* and 2.8-fold in Δ*opaRΔrpoS* compared to the wild-type strain at the log growth phase (Fig. 3a). Moreover, *prtA* expression decreased 1.6-fold in Δ*opaR*, 2-fold in Δ*rpoS* and 2.4-fold in Δ*opaRΔrpoS* compared to the wild-type strain at the stationary growth phase (Fig. 3b). These data suggest that *OpaR* promotes *prtA* expression after the log growth phase while *RpoS* enhances the expression of *prtA* after the stationary phase. We then amplified the promoter region of *prtA* and constructed *luxAB* promoter fusion strains to monitor *prtA* gene expression using a luciferase activity assay. Luciferase activity decreased both at the log (Fig. 3c) and at the stationary growth phases (Fig. 3d) when the *opaR* gene was deleted. These results indicate that *OpaR* regulates *prtA* through the *prtA* promoter region. Comparing the qPCR and luciferase activity results of Δ*rpoS* and Δ*opaRΔrpoS* strains, we found that *RpoS* did not regulate *prtA* expression through the *prtA* promoter region that we amplified. *RpoS* would promote *prtA* expression indirectly. However, the hierarchy and correlation of the two global regulators have not been clarified in *V. parahaemolyticus*.

### OpaR and RpoS enhance the amount of extracellular PrtA and the PrtA protease activity of *V. parahaemolyticus*

Extracellular protease would interact with the host cell or the ecological environment. Therefore, we investigated the
We also examined the PrtA protease activity of V. parahaemolyticus wild-type (VP93) and mutant strains (ΔopaR, ΔrpoS and ΔopaRΔrpoS). The results showed that PrtA protease activity of the mutant strains ΔopaR, ΔrpoS and ΔopaRΔrpoS decreased more than 2-fold at late-log (Fig. 4b) and stationary phases (Fig. 4c) as compared with that of the wild-type (VP93). These data suggested that OpaR and RpoS affect not only the amount of extracellular PrtA protein but also the PrtA protease activity of VP93.

**OpaR binds to the promoter region of prtA**

Results in this study demonstrated that OpaR upregulated *prtA* expression. Therefore, we speculated whether OpaR binds to the *prtA* promoter region to regulate *prtA*. EMSA was used to determine the interaction between His-OpaR and the *prtA* promoter region. We made searches of the *prtA* promoter region using the matrices ‘paster’ tool of the RetrieveSeq program with the MQSR position frequency matrix [18]. This bioinformatic tool predicted that the putative OpaR binding sites were located at positions −361 to −229 and −145 to −3 from the *prtA* translation start site, annotated PrtA-1 and PrtA-2, respectively (Fig. 5a). DIG-labelled PrtA-1 and PrtA-2 were mixed with His-OpaR during the binding reactions, respectively. The gel shift assay results showed that OpaR specifically binds to both PrtA-1 and PrtA-2 (Fig. 5b). In the competition assay, after adding fragments not labelled with DIG to compete with the DNA-OpaR complex, the binding

**Fig. 3.** OpaR and RpoS regulated gene expression of *prtA*. A quantitative survey of *prtA* expression in VP93, ΔopaR, ΔrpoS and ΔopaRΔrpoS strains was conducted via qRT-PCR at (a) log phase (OD_{600} of 0.5) and (b) stationary phase (2 h after reaching an OD_{600} of 2). Luciferase activity was measured in VP93, ΔopaR, ΔrpoS and ΔopaRΔrpoS strains at (c) log phase (OD_{600} of 0.5) and (d) stationary phase (2 h after reaching an OD_{600} of 2). Values are the mean of three independent experiments. Error bars indicate the standard deviation (*P<0.01).
signals decreased or disappeared (Fig. 5b, lane 5). The EMSA results demonstrated the specificity of the DNA-OpaR complex and indicated OpaR regulated \( \text{prtA} \) expression by binding to the promoter region directly.

To confirm the specific binding sequence of OpaR, we performed a DNase I protection assay as described in Materials and Methods. The 480-bp \( \text{prtA} \) promoter region was amplified with 6-FAM-labelled sense and antisense strand primer sets separately. The 6-FAM-labelled sense and antisense probes were bound to His-OpaR and digested with DNase I, respectively. The digested DNA pool was then analysed using capillary electrophoresis. We compared the capillary electrophoresis pattern of 6-FAM-labelled probes with and without His-OpaR protein, and found that two regions of the \( \text{prtA} \) promoter were protected by the OpaR protein. OpaR binds to 24 bp of \( \text{PrtA-1} \), from \( -269 \) to \( -246 \) (5’-ATATTAGAATAATTACTCATA-3’) (Fig. 6a, b), and 21 bp of \( \text{PrtA-2} \) from \( -88 \) to \( -68 \) (5’-TAGCTGAGTGAATCATGTATA-3’) (Fig. 6c, d), relative to the \( \text{prtA} \) translation start site. The results of the capillary electrophoresis patterns of the 6-FAM-labelled antisense strand were identical to those of the sense strand.

**Analysis of the intergenic region of \( \text{prtA} \) and upstream gene \( \text{ldh} \)**

The \( \text{ldh} \) gene is located upstream of \( \text{prtA} \). The length of the intergenic region between \( \text{prtA} \) and \( \text{ldh} \) is 469 bp (Fig. 7a). We identified the transcription start site of \( \text{prtA} \) and \( \text{ldh} \) via ARF-TSS [29] as described in Materials and Methods. The transcription start site of \( \text{prtA} \) (+1) and \( \text{ldh} \) (+1) were located at \( -198 \) from the \( \text{prtA} \) translation start site, and \( -76 \) from the \( \text{ldh} \) translation start site, respectively. The prediction of \( -35 \) and \( -10 \) promoter sequences was determined using the SoftBerry BPROM program (http://www.softberry.com). Predicted \(-35\) and \(-10\) promoter sequences were TAGAAT(–35)-N18-ATTTAACAT(–10) for \( \text{prtA} \) and TTATTT(–35)-N12-TGTATTAAAT(–10) for \( \text{ldh} \). The SoftBerry BPROM program also predicted that the \( \sigma^{70} \) recognition sequence covered the

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**Fig. 4.** The absence of OpaR or RpoS alone decreased the amount of extracellular PrtA and PrtA protease activity of VP93. (a) The amount of extracellular and intracellular PrtA from wild-type VP93, \( \Delta \text{opaR} \), \( \Delta \text{rpoS} \) and \( \Delta \text{opaR} \Delta \text{rpoS} \) was detected using Western blotting at the log phase (OD\(_{600}\) of 0.5), late-log phase (OD\(_{600}\) of 2), stationary phase (2 h after reaching an OD\(_{600}\) of 2) and late-stationary phase (4 h after reaching an OD\(_{600}\) of 2). PrtA protease activity of wild-type VP93, \( \Delta \text{opaR} \), \( \Delta \text{rpoS} \), \( \Delta \text{opaR} \Delta \text{rpoS} \) and \( \Delta \text{prtA} \) was measured at (b) the late-log phase (OD\(_{600}\) of 2) and (c) stationary phase (2 h after reaching an OD\(_{600}\) of 2). Values are the mean of three independent cultures. Error bars indicate the standard deviation (*\( P<0.01 \)).
−10 box region of \(ldh\) and \(prtA\). PRODIGAL [33] was used to define the ribosomal binding site (RBS) sequence. The putative RBSs were AAC, –15 from the \(prtA\) translation start site, and GAAGA, –4 from the \(ldh\) translation start site. We also investigated the correlation of OpaR and \(ldh\) using real-time qPCR. We found that \(ldh\) is expressed about 5-fold more at the early-log phase compared to other growth phases in VP93 (Fig. 7b). Expression of \(ldh\) decreased about 2.5-fold in \(\Delta\)opaR (Fig. 7c). These data demonstrated that OpaR upregulates the expression of \(ldh\) at the early-log phase.

**DISCUSSION**

TDH and TRH are two well-recognized virulence factors of *V. parahaemolyticus*. The haemolytic activity of TDH can be blocked after incubation with gangliosides GT1 and GD1a. However, GT1 and GD1a cannot neutralize the haemolytic activity of PrtA. PrtA has also been shown to cause obvious haemorrhaging in the abdominal and thoracic cavities of mice [12]. Therefore, PrtA is a novel putative virulence factor that has different characteristics from TDH and TRH. Recently, a reporter gene assay indicated that the expression of \(prtA\) is regulated by lateral flagella regulator, LafK, and induced by solid surfaces. Therefore, PrtA is considered to belong to the surface-sensing regulon [34]. In this study, we cultured VP93 and mutants in liquid medium to observe the expression and regulation of \(prtA\). The experimental results indicate that PrtA is the extracellular protease activity dominator in the swimming cell lifestyle of VP93 while growing to high cell density. Microarray analysis in *V. parahaemolyticus* solid surface culture revealed that OpaR regulates 210 genes negatively and 113 genes positively (4-fold). Genes upregulated by OpaR include genes that code for capsular polysaccharides, T6SS2, c-di-GMP modulation, sRNA, Rhs-family proteins, competency, polyhydroxyalkanoates (PHA) synthesis and sodium/solute symporter. On the other hand, repressed genes by OpaR were classified as coding for the surface-sensing regulon, T3SS1, T6SS1, iron transporters, c-di-GMP modulation and amino acid transporters [32]. The RNA-Seq results for *V. parahaemolyticus* strain BB22 reported that OpaR also controls nine transcription factors [35]. However, we showed that OpaR also enhances the expression of \(prtA\) by binding to the promoter region in liquid culture.

Previous reports have shown that RpoS promotes the expression of *V. cholerae* HAP. It also coordinates with the QS regulator of *V. cholerae*, HapR, to regulate HAP [36]. We showed that RpoS not only promotes the expression of \(prtA\) at the transcription level, but also affects the amount of extracellular PrtA. Moreover, LuxR in *V. harveyi* has both activator and repressor functions [37]. The binding motif of LuxR is different when it acts as an activator or a repressor. The conserved binding sequence of the QS regulator in vibrios remains unclear [38]. In this study, we observed that there are two OpaR-protected regions located within the \(prtA-ldh\) intergenic sequence. Results in this study indicate
that *prtA* is expressed at the log growth phase and *ldh* at the early-log growth phase. The gene expression of *prtA* and *ldh* were upregulated by OpaR.

There has been no comprehensive analysis of the promoter region in *V. parahaemolyticus*. Thus, we predicted the promoter region of *prtA* and *ldh* using BPROM (http://www.softberry.com) and NNPP 2.2 (http://www.fruitfly.org/seq_tools/promoter.html) [39]. BPROM and NNPP 2.2 were developed for the recognition of *E. coli* σ^70^ promoters. BPROM has 80% prediction accuracy. The −10 box sequence is thought to be an intrinsic characteristic of the σ promoter in bacteria [40]. Browning and Busby reported that the −10 element region of the bacterial promoter would be recognized as a ssDNA [41]. BPROM shows that *ldh* shared the −10 box sequence on the complementary strand with *prtA*. We also found that the σ^70^ recognition sequence covered the −10 box sequence based on the predictions of BPROM and BacPP (http://www.bacpp.bioinfoacs.com/ferramenta) [42]. BacPP showed that the recognition sequence of RpoD (σ^70^) located at −259 to −191 from the *prtA* translation start site. BPROM also predicted that there is a RpoD recognition sequence at positions −238 to −231 from *prtA* translation start site.

From the *E. coli* promoter database, a published study found that about 14% forward promoters overlapped with a reverse promoter in the opposite direction. These promoters can present head-to-head or tail-to-tail arrangements [43]. Overlapping promoters of two opposite genes in *V. cholerae* have also been reported. The authors indicated that a virulence gene, igrA, in *V. cholerae* shares the promoter region
of its upstream opposite gene, \textit{irgB}. The transcription factor Fur regulated \textit{irgA} by binding to the intergenic region of \textit{irgA}–\textit{irgB} [44]. Therefore, we presumed that \textit{prtA} and \textit{ldh} could share the promoter region in VP93.

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\textbf{Conflicts of interest}
The authors declare that there are no conflicts of interest.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{The role of OpaR in the VP93 \textit{ldh}–\textit{prtA} intergenic region. (a) Nucleotide positions are numbered relative to the translational initiation site of \textit{prtA}. The transcriptional start sites of \textit{prtA} and \textit{ldh} are labelled +1. The \textit{−10} and \textit{−35} promoter sequences and ribosomal binding sites of \textit{prtA} and \textit{ldh} are shown in red and blue, respectively. Predicted OpaR binding sites, \textit{PrtA-1} and \textit{PrtA-2}, are indicated by a wavy underline. The primer sets used to amplify \textit{PrtA-1} and \textit{PrtA-2} are shown with black arrows. OpaR protection sequences of footprinting are boxed. SD, Shine–Dalgarno sequence. (b) Gene expression level of \textit{ldh} was measured using qRT-PCR at the early-log phase (OD$_{600}$ of 0.1), log phase (OD$_{600}$ of 0.5), late-log phase (OD$_{600}$ of 2) and stationary phase (2 h after reaching an OD$_{600}$ of 2). (c) At the early-log phase (OD$_{600}$ of 0.1), qRT-PCR was conducted to quantify the expression of \textit{ldh} in VP93 and \textit{ΔopaR}.}
\end{figure}

\begin{thebibliography}{9}
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\end{thebibliography}


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