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The quorum sensing regulator OpaR is a repressor of polar flagellum genes in *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus **possesses two types of flagella: a single polar flagellum (Pof) for swimming and the peritrichous lateral flagella (Laf) for swarming. Expression of Laf genes has previously been reported to be regulated by the quorum sensing (QS) regulators AphA and OpaR. In the present study, we showed that OpaR, the QS regulator at high cell density (HCD), acted as a negative regulator of swimming motility and the transcription of Pof genes in *V. parahaemolyticus*. OpaR bound to the promoter-proximal DNA regions of *flgAMN*, *flgMN*, and *flgBCDEFGHIJ* within the Pof gene loci to repress their transcription, whereas it negatively regulates the transcription of *flgKL-flaC* in an indirect manner. Thus, this work investigated how QS regulated the swimming motility via direct action of its master regulator OpaR on the transcription of Pof genes in *V. parahaemolyticus*.**

Keywords: *Vibrio parahaemolyticus*, polar flagellum, quorum sensing, OpaR

Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium that naturally inhabits marine ecosystems. It possesses dual flagellar systems, a single polar flagellum (Pof) for swimming in liquids and the peritrichous lateral flagella (Laf) for swarming over surfaces or in viscous liquids (McCarter, 2004). The Pof is continuously produced, whereas the Laf are expressed only under certain conditions (McCarter and Silverman, 1989; Kawagishi *et al.*, 1996). In addition, unlike that of Pof genes, the expression of Laf genes is highly regulated by numerous transcriptional regulators, including LafK (Gode-Potratz *et al.*, 2011), SwrZ (Jaques and McCarter, 2006), SwrT (Jaques and McCarter, 2006), H-NS (Wang *et al.*, 2018), VapH (Park *et al.*, 2005), CalR (Gode-Potratz *et al.*, 2010), and the quorum sensing (QS) regulators AphA and OpaR (Jaques and McCarter, 2006; Wang *et al.*, 2013a; Lu *et al.*, 2019). *Vibrio parahaemolyticus* swimming motility has been shown to be activated by OxyR (Chung *et al.*, 2016), AphA (Wang *et al.*, 2013a), and ToxR (Chen *et al.*, 2018), but detailed information on its regulatory mechanisms is lacking. Overall, regulation of Pof genes is far from elucidated.

QS is a cell-to-cell communication process used by bacteria to regulate gene expression via detection of signaling molecules called autoinducers, which are produced by bacteria in response to changes in bacterial cell density (Ng and Bassler, 2009). QS generally uses downstream master regulators to control gene expression (Ng and Bassler, 2009). It has been shown that AphA and OpaR are the two master QS regulators in *V. parahaemolyticus*, and their highest expression levels occur at low cell density (LCD) and high cell density (HCD), respectively (Sun *et al.*, 2012; Zhang *et al.*, 2012). Both individually and together, they regulate multiple cellular pathways in *V. parahaemolyticus*, including virulence factor production, motility, and biofilm formation (Henke and Bassler, 2004; van Kessel *et al.*, 2013; Wang *et al.*, 2013a, 2013b; Lu *et al.*, 2018, 2019).

The swarming motility regulatory mechanisms by AphA and OpaR have been elucidated in *V. parahaemolyticus* (Lu *et al.*, 2019), but the swimming motility mechanisms of these two regulators are still lacking. In the present study, we showed that OpaR bound to the promoter-proximal DNA regions of *flgAMN*, *flgMN*, and *flgBCDEFGHIJ* within the Pof gene loci to repress their transcription, whereas it represses *flgKL-flaC* transcription in an indirect manner. Thus, OpaR repressed swimming motility by acting directly on the regulatory regions of Pof genes.

Materials and Methods

Bacterial strains and growth conditions

The pandemic *V. parahaemolyticus* strain RIMD 2210633 was used as the wild-type (WT) strain (Makino *et al.*, 2003). Nonpolar *opaR* deletion mutant derived from the WT strain, termed *ΔopaR*, and the complementary mutant strain *ΔopaR*/pBAD33-*opaR*, termed C-*ΔopaR*, were constructed as described in our previous studies (Zhang *et al.*, 2012, 2019). The empty pBAD33 was also introduced into WT and *ΔopaR* to generate WT/pBAD33 and *ΔopaR*/pBAD33 to counteract the effects of arabinose and chloramphenicol on bacterial growth and physiology (Zhang *et al.*, 2019).

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Glycerol stocks of bacterial cells were inoculated into 5 ml of 2.5% (w/v) Bacto heart infusion (HI) broth (BD Biosciences) at 37°C with shaking at 200 rpm for 12–14 h. The resulting cell cultures were diluted 50-fold into 5 ml of fresh HI broth and then cultured at 37°C to reach an optical density at 600 nm (OD_{600}) value of approximately 1.0 to 1.4. The cell cultures were then diluted 1,000-fold into 10 ml OTN broth (1% [w/v] Oxoid tryptone [Oxoid] supplemented with 2% [w/v] NaCl [Merck]) for a third round of incubation and then harvested at the required cell densities. When necessary, the medium was supplemented with 100 μg/ml gentamicin.

Swimming motility assay

For the swimming motility assay (Wang *et al.*, 2013a), 2 μl of cell culture from the second inoculation in HI broth was inoculated into the semi-solid swim plates (1% Oxoid tryptone, 2% NaCl [Merck], and 0.5% Difco Noble agar [BD Biosciences]). The diameter of the area covered by the swimming bacteria was measured per hour after incubation at 37°C.

Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen). The cDNA was generated from 2 μg of each RNA sample using the FastKing First Strand cDNA Synthesis Kit (Tiangen Biotech) according to the manufacturer's instructions. The qPCR assay was performed using a LightCycler system (Roche) with SYBR Green master mix (Gao *et al.*, 2011). The relative mRNA levels of each target gene were determined using the $2^{-\Delta\Delta Ct}$ method (Gao *et al.*, 2011). A mean ratio of two was used as the cutoff value for statistical significance. Experiments were performed independently at least three times. Primers used in the present study are listed in Table 1.

Preparation of 6× His-tagged OpaR (His-OpaR) protein

The entire coding region of *opaR* was cloned into the pET28a plasmid, and then transformed into *Escherichia coli* BL21DE3 cells for protein (His-OpaR) expression (Kleber-Janke and Becker, 2000). Expression and purification of His-OpaR was carried out as described previously (Zhang *et al.*, 2012). The purity of the purified His-OpaR was analyzed by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis.

Electrophoresis mobility shift assay (EMSA)

The EMSA was performed as previously described (Zhang *et al.*, 2012, 2017). Briefly, the 5′-ends of the promoter DNA regions of each target gene were labeled with [γ-^{32}P]-ATP. The EMSA was performed in a 10 μl reaction volume containing binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl; pH 7.5, and 0.05 mg/ml salmon sperm DNA), labeled DNA probe (100–200 CPM/μl), and increasing amounts of His-OpaR. Two controls were included: (1) cold probe as a specific DNA competitor (unlabeled corresponding promoter DNA fragments) and (2) nonspecific protein competitor (rabbit anti-F1-protein polyclonal antibodies). The EMSA reactions were incubated at room temperature for 20 min, and the binding products were analyzed in native 4% (w/v) polyacrylamide gels. The results were detected by autoradiography after exposure to Fuji Medical X-ray film (Fujifilm Corp.).

DNase I footprinting

For DNase I footprinting (Zhang *et al.*, 2012, 2017), promoter DNA regions with a single ^{32}P-labeled end were generated by

Table 1. Oligonucleotide primers used in this study

Target	Primers (forward/reverse, 5′-3′)
qPCR	
flgM (VP0771)	ATTCAAGTGCGACATCAAG/CGGAGAAGCTGCCATATC
flgA (VP0772)	ATTGCCCTGAACCACTCG/ATCGGTACTGAAGAGGAGACAC
flgB (VP0775)	ACAAGGCACTAGGCATCC/GACCATCTGTTCGGCTAAG
flgK (VP0785)	GCCGTCAGTCAGTGATTC/GTAGAGGACAGGTTGAGTTC
LacZ fusion	
flgM (VP0771)	GCGCTCTAGATGTCCCTGTTCGTGTCTC/GCGCGAATTCATGTCGCACTTGAATCGTTAC
flgA (VP0772)	GCGCGTCGACTTTCTTGCTCCGCTTGTG/GCGCGAATTCCTGATTATCTGTTGCCGAATGC
flgB (VP0775)	GCGCGTCGACTTAGGTCGTGGTCTTTCG/GCGCGAATTCTTGCCGCTTGTAACTCCTTG
flgK (VP0785)	GCGCGTCGACAACCATCATTGCTTCTTG/GCGCGAATTCTGCTTGTAGAGGACAGGTTGAG
Protein expression	
opaR	AGCGGGATCCATGGACTCAATTGCAAAGAG/AGCGAAGCTTTTAGTGTTCGCGATTGTAG
EMSA	
flgM (VP0771)	CGTAGCGTTCGTCTTGGTG/GCCATGTTTAACCTTTGCCTTG
flgA (VP0772)	TTGCCAGGCTCAAATCAATTAC/AATGCCGATAGACTTAGCGTAC
flgB (VP0775)	AATCCAGGTATCATTTACAAGC/TGGATGCCTAGTGCCTTGTC
flgK (VP0785)	AACCATCATTGCTTCTTG/TGCTTGTAGAGGACAGGTTGAG
DNase I footprinting	
flgM (VP0771)	CGTAGCGTTCGTCTTGGTG/TTCCGCAATGTCAGTCACTATG
flgA (VP0772)	VTTGCCAGGCTCAAATCAATTAC/TGACGGGGATTCTTGACTCG
flgB (VP0775)	GCTAATCTGTTGTCGGACTTTG/TGGATGCCTAGTGCCTTGTC
flgK (VP0785)	TTGCAGCAACGTGGAGATTC/TGAGCAGTAAGCACACTTTGAG

PCR with either sense or antisense primers, end-labeled, and purified using QiaQuick columns (Qiagen). DNA binding was performed in a 10 µl reaction volume containing binding buffer (the same used for EMSA), labeled DNA fragment (2–5 pmol), and increasing amounts of His-OpaR. The reactions were incubated at room temperature for 30 min. Prior to digestion, 10 µl of a Ca^{2+}/Mg^{2+} solution (5 mM $CaCl_2$ and 10 mM $MgCl_2$) was added to each reaction and incubated for 1 min at room temperature. Optimized RQ1 RNase-Free DNase I (Promega) was added to each reaction mixture and then incubated at room temperature for 40–90 sec. The reaction was quenched by adding 9 µl of stop solution (200 mM NaCl, 30 mM EDTA, and 1% SDS), followed by incubation for 1 min at room temperature. The partially digested DNA samples were extracted with phenol/chloroform, precipitated with ethanol, and analyzed on 6% polyacrylamide/8 M urea gels. Protected regions were identified by comparison with the sequence ladder. The templates for Sanger sequencing were the same as the DNA fragments used in the DNase I footprinting assay. Sanger sequencing was carried out using an AccuPower & Top DNA Sequencing Kit (Bioneer) according to the manufacturer's instructions. Radioactive species were detected by autoradiography after exposure to Fuji Medical X-ray film (Fujifilm Corp.).

Statistical methods

Swimming motility and qPCR assays were carried out with at least three independent bacterial cultures, and the values were expressed as the mean ± standard deviation (SD). Paired Student's *t*-tests were used to calculate statistically significant differences. $P < 0.01$ was considered statistically significant. Data for EMSA and DNase I footprinting assays were collected from at least two independent biological replicates.

Results

OpaR represses swimming motility

Regulation of swimming motility by the QS regulator OpaR was investigated, and the results showed that swimming capacity was significantly increased in *ΔopaR* compared with WT (Fig. 1) at all time points tested. In addition, the diameter of the area covered by swimming bacterial cells increased with cultivation time for both the WT and *ΔopaR* strains. These observations suggested that OpaR was a repressor of swimming motility in *V. parahaemolyticus*.

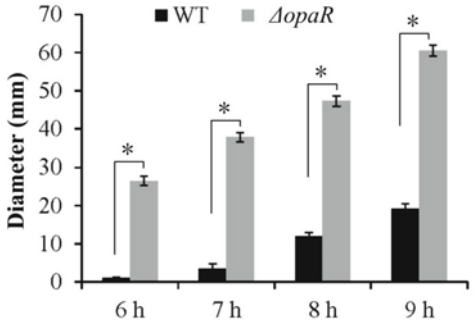

Fig. 1. Regulation of swimming motility by OpaR. Swimming was evaluated by measuring the diameter of the area covered by swimming bacterial cells in semi-solid swimming plates. Results are expressed as the mean ± SD and were analyzed by paired Student's *t*-test. The asterisk (*) represents $P < 0.01$.

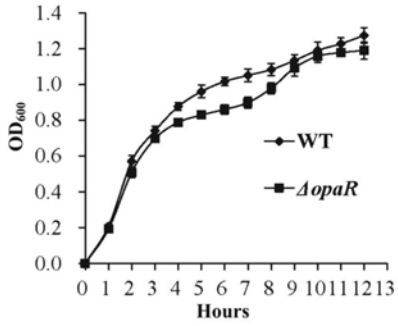

Fig. 2. Growth curves of WT and *ΔopaR*. The WT and *ΔopaR* strains were grown in the OTN broth at 37°C with shaking at 200 rpm, and the OD_{600} values of each strain were monitored at 1-h intervals. Experiments were performed at least two times with three replicates per trial for each strain.

Growth of WT and *ΔopaR* strains

The WT and *ΔopaR* strains were grown in OTN broth at 37°C with shaking at 200 rpm, and OD_{600} values of each strain were monitored at 1 h intervals to create growth curves. As shown in Fig. 2, indistinguishable growth rates were ob-

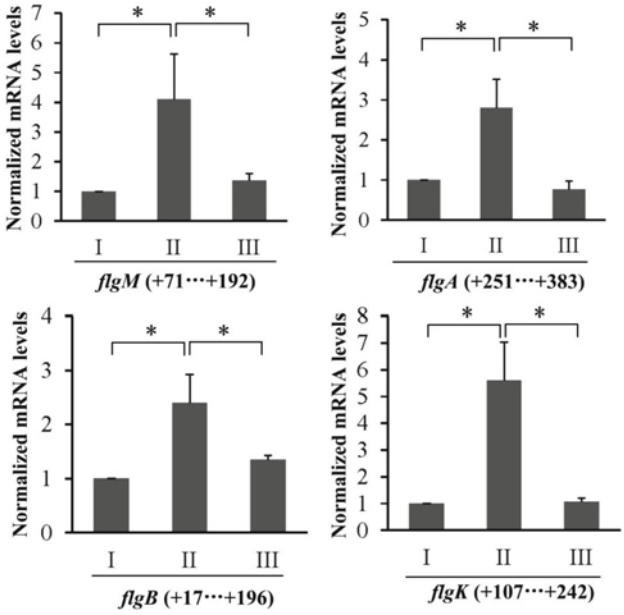

Fig. 3. Negative regulation of Pof genes by OpaR. Negative and positive numbers represent the nucleotide positions upstream and downstream of each target gene. The relative mRNA levels of each target gene were compared between WT/pBAD33, *ΔopaR*/pBAD33, and C-*ΔopaR*. I, II, and III represent WT/pBAD33, *ΔopaR*/pBAD33, and C-*ΔopaR*, respectively. The asterisk (*) represents $P < 0.01$, which was determined by a paired Student's *t*-test.

served for these two strains in the medium, suggesting that deletion of *opaR* did not affect the growth of *V. parahaemolyticus* in OTN broth. Bacterial cells were harvested at the early exponential stage (an OD_{600} value of approximately 0.8) for the following biochemical assays.

Selection of target genes

The Pof gene system comprises approximately 60 genes, and most of these genes are located in two regions, containind at least 11 operons: *flgAMN*, *flgMN*, *cheVR*, *flgBCDEFGHIJ*, *flgKL-flaC*, *flgKL*, *flaDE*, *flaAGHIJK*, *flaKLM*, *fliEFGHIJ-KLMNOPQR-flhB*, and *flhAFG-fliA-cheYZAB*-ORF1-ORF2-*cheW*-ORF3 (Kim and McCarter, 2000; McCarter, 2001). The 500 bp DNA regions upstream of the first genes in these operons were downloaded from the WT genome. The DNA-binding box of OpaR (Zhang *et al.*, 2012) was used to predict the presence of OpaR box-like sequences within the DNA regions using the matrix scan tool (van Helden, 2003). At a cutoff value score of 6 (Zhang *et al.*, 2012), no OpaR box-like sequences were detected for any DNA sequence tested (data not shown).

The OpaR box has been proven to be useful for the prediction of direct OpaR targets, but because it was created from LuxR homolog binding sites, using this box may lead to potential binding sites being assigned lower scores and some potential OpaR sites to be missed (Zhang *et al.*, 2012; Kernell Burke *et al.*, 2015). Thus, the first genes in the *flgAMN*, *flgMN*,

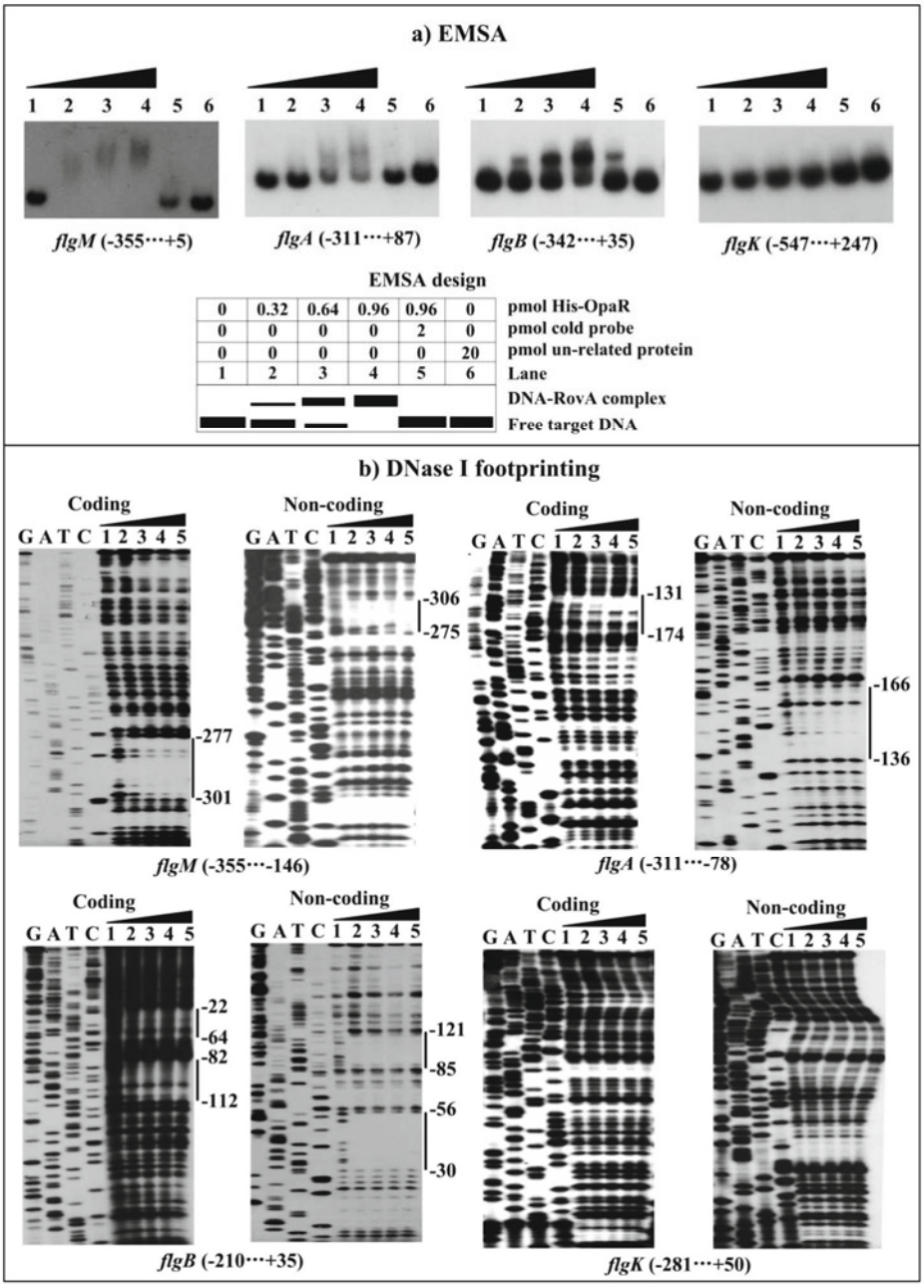

Fig. 4. Binding of His-OpaR to the promoter DNA regions of Pof genes. Negative and positive numbers represent the nucleotide positions upstream and downstream of each target gene. (A) EMSA. The entire promoter DNA region of each target gene was incubated with increasing amounts of His-OpaR and then subjected to 6% (w/v) polyacrylamide gel electrophoresis. The EMSA design is shown below. (B) DNase I footprinting. Lanes G, A, T, and C represent Sanger sequencing reactions. Labeled coding or non-coding DNA probes were incubated with increasing amounts of purified His-OpaR (Lanes 1, 2, 3, 4, and 5 contained 0, 3, 6, 9, and 12 pmol, respectively), and were subjected to DNase I footprinting. The protected regions are indicated with vertical bars.

flgBCDEFGHIJ, and *flgKL-flaC* operons were randomly selected as target genes for subsequent gene regulation studies.

OpaR represses the transcription of Pof genes

The qPCR assays were employed to detect differences in mRNA levels of Pof genes between WT/pBAD33, *ΔopaR*/pBAD33, and C-*ΔopaR*. As shown in Fig. 3, the mRNA level of each target gene was significantly increased in *ΔopaR*/pBAD33 relative to WT/pBAD33 and C-*ΔopaR*, whereas expression levels of each target gene were restored in C-*ΔopaR*. These results suggested that OpaR repressed Pof gene transcription in *V. parahaemolyticus*.

Direct binding of His-OpaR to the regulatory DNA regions of Pof genes

The promoter-proximal DNA region of each target gene was amplified and subjected to EMSA with purified His-OpaR. As shown in Fig. 4A, His-OpaR was able to bind to the promoter-proximal DNA regions of *flgM*, *flgA*, and *figB* in a dose-dependent manner, but it was unable to bind to the promoter-proximal DNA region of *flgK* at all protein amounts tested. As further determined by DNase I footprinting (Fig. 4B), His-OpaR protected single DNA regions located from 306 to 275 bp and from 174 to 131 bp within the upstream DNA regions of *flgM* and *flgA*, respectively, against DNase I digestion, whereas it protected two different DNA regions from 121 to 82 bp and 64 to 22 bp upstream of *flgB*. These protected regions were considered OpaR binding sites. However, no His-OpaR protected DNA regions were detected in the upstream DNA region of *flgK*. Thus, OpaR directly repressed the transcription of *flgM*, *flgA*, and *flgB*, whereas it indirectly repressed *flgK* transcription.

Discussion

Vibrio parahaemolyticus swimming motility, powered by the Pof, is a mode of movement of individual bacterial cells in liquid environments (McCarter, 2004). In contrast, swarming motility, powered by the Laf, is a process by which bacteria move on surfaces or in viscous liquids in a coordinated manner (McCarter, 2004). More studies have focused on the Laf by far, especially those that involve the investigation of

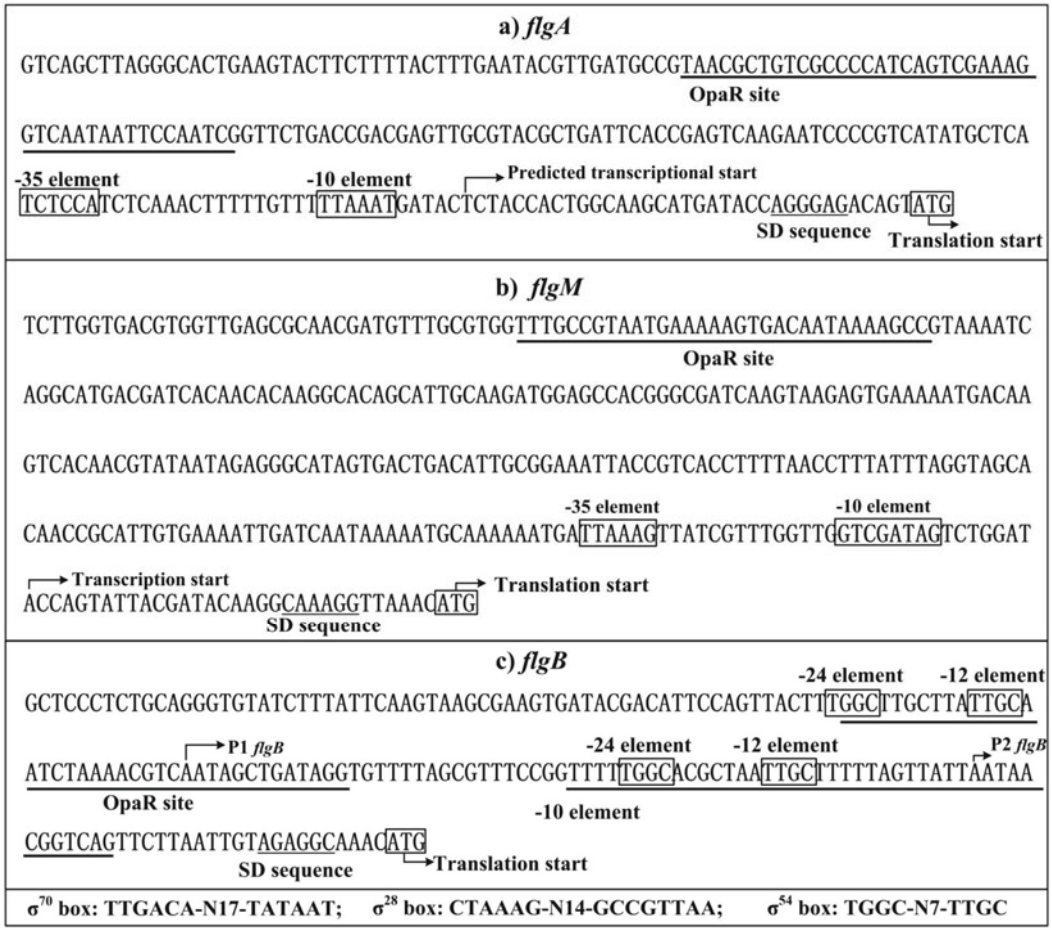

Fig. 5. Organization of promoter DNA regions of Pof genes. The promoter DNA regions of selected target genes were derived from *V. parahaemolyticus* RIMD 221063. The translation and transcription start sites are shown with bent arrows. The predicted core promoters (-10 and -35 or -12 and -24 elements) are boxed. The OpaR binding sites and the SD sequences are underlined. The σ^{70}, σ^{28}, and σ^{54} boxes are shown at the bottom of the Figure.

its roles in biofilm formation and the regulation of its coding genes (McCarter and Silverman, 1989; McCarter, 2004; Park *et al.*, 2005; Jaques and McCarter, 2006; Gode-Potratz *et al.*, 2010; Wang *et al.*, 2013a, 2018; Chung *et al.*, 2016; Lu *et al.*, 2019). However, more than 50 gene products are involved in assembling a functional Pof, and thus, bacteria must also employ complex regulatory systems to control Pof gene expression (McCarter, 2001). AphA, the QS regulator at LCD, plays a positive role in regulating swimming in *V. parahaemolyticus*, and deletion of *aphA* significantly decreases swimming motility capacity relative to WT (Wang *et al.*, 2013a). ToxR also acts as an activator of swimming motility in *V. parahaemolyticus*, and the swimming motility capacity of the *ΔtoxR* strain is dramatically decreased relative to WT (Chen *et al.*, 2018). Swimming capacity is also significantly inhibited in the *ΔoxyR* strain relative to WT, suggesting that OxyR is also a positive regulator of swimming motility in *V. parahaemolyticus* (Chung *et al.*, 2016). In addition, the VcrD1 protein, a component of the *V. parahaemolyticus* type III secretion system 1, plays a key role in flagellar morphogenesis via regulation of the expression and secretion of flagellar components (Noh *et al.*, 2015).

In the present work, we showed that OpaR, the QS regulator at HCD, acted as a negative regulator of swimming motility and Pof gene transcription in *V. parahaemolyticus*. OpaR bound to the promoter-proximal DNA regions of *flgAMN*, *flgMN*, and *flgBCDEFGHIJ* within the Pof gene loci to repress their transcription, whereas it negatively regulated the transcription of *flgKL-flaC* in an indirect manner. Thus, the repression of swimming motility by OpaR in *V. parahaemolyticus* occurred via the direct and negative regulatory actions of OpaR on the transcription of Pof genes. OpaR homologs in other *Vibrio* species, including SmcR, VtpR, HapR, and LuxR, have been demonstrated to be involved in regulating Pof biosynthesis (Nielsen *et al.*, 2006; Hasegawa and Hase, 2009; Kim *et al.*, 2012; Yang and Defoirdt, 2015). In *V. vulnificus* and *V. tubiashii*, SmcR and VtpR repress flagellar biosynthesis and swimming motility (Hasegawa and Hase, 2009; Kim *et al.*, 2012), whereas LuxR and HapR activate the expression of flagellar genes and swimming motility in *V. harveyi* and *V. cholerae* (Nielsen *et al.*, 2006; Yang and Defoirdt, 2015). Thus, regulation of flagellar genes by the master QS regulator at HCD may be dependent on genetic background.

Many reports have demonstrated that the two master QS regulators AphA and OpaR coordinately and inversely regulate the transcription of their target genes in *V. parahaemolyticus* RIMD2210633 during the transition from LCD to HCD (Sun *et al.*, 2012; Zhang *et al.*, 2012, 2019; Wang *et al.*, 2013b; Zhou *et al.*, 2013; Lu *et al.*, 2019). Transcriptional regulation of Pof genes by AphA and OpaR is another example of this phenomenon. The motility mediated by the Pof is closely related to biofilm formation and virulence (Enos-Berlage *et al.*, 2005; Yildiz and Visick, 2009; Yang and Defoirdt, 2015). Interestingly, it has been suggested that the Pof is involved in the initial stage of biofilm formation, but its biosynthesis is repressed after the biofilm is formed (Yildiz and Visick, 2009; Zhu *et al.*, 2013). OpaR activate the expression of biofilm-associated genes at HCD (McCarter, 1998; Enos-Berlage and McCarter, 2000). The possible benefits of OpaR-mediated repression of Pof gene expression are not yet clear. Down-regulation of Pof genes by the master QS regulator OpaR at HCD suggests that swimming motility is not as important during the late growing period or during the later stage of infection. Inhibiting the expression of Pof genes could help bacteria conserve limited nutrients.

The promoter organization of *flgAMN*, *flgMN*, and *flgBCDEFGHIJ* were reconstructed herein by collecting data on the translation/transcription start sites (Kim and McCarter, 2000; McCarter, 2001), core promoter elements (-10/-35 and -12/-24), OpaR binding sites, and Shine-Dalgarno (SD) sequences (Fig. 5). The *flgMN* operon is part of the larger *flgAMN* operon, that is, the *flgMN* genes can also be transcribed from the *flgAMN* promoter (Kim and McCarter, 2000; McCarter, 2001). A σ^{28}-dependent promoter was previously identified for the initiation of *flgMN* (also seen in Fig. 5B) (Kim and McCarter, 2000), but no transcription start sites have been determined for *flgAMN*. However, a σ^{70}-dependent promoter was predicted for *flgAMN* using the online Softberry tool (http://linux1.softberry.com/berry.phtml) (Fig. 5A). The OpaR binding site for *flgAMN* or *flgMN* was located far upstream of the -35 element, which was an unusual position for a regulator that repressed its target genes. There may be unknown regulators that activated the transcription of *flgAMN* and *flgMN*, and the binding of OpaR may interfere with their functions. Two σ^{54}-dependent promoters, named P1 and P2, have been identified upstream of *flgBCDEFGHIJ* that initiate its transcription (Fig. 5C) (Kim and McCarter, 2000). The two OpaR binding sites for *flgBCDEFGHIJ* entirely overlapped each of the promoters (Fig. 5C). Thus, OpaR repressed the transcription of *flgBCDEFGHIJ*, possibly via direct interference with RNA polymerase. In addition, the binding of OpaR to these two sites may lead to the occurrence of hairpins, thereby causing interference with the production of mRNAs.

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Conflict of Interest

The authors have no conflicts of interest to report.

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