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. Overall, regulation of Pof genes is far from elucidated.

**Vibrio parahaemolyticus** has been shown to be regulated by the QS regulators AphA and OpaR, 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 swimming 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 negatively regulates the transcription of *flgKL-flaC* in an indirect manner.

**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 \(\Delta\text{opaR}\), and the complementary mutant strain \(\Delta\text{opaR}/\text{pBAD33-opaR}\), termed C-\(\Delta\text{opaR}\), were constructed as described in our previous studies (Zhang et al., 2012, 2019). The empty \(\text{pBAD33}\) was also introduced into WT and \(\Delta\text{opaR}\) to generate WT/\(\text{pBAD33}\) and \(\Delta\text{opaR}/\text{pBAD33}\) to counteract the effects of arabinose and chloramphenicol on bacterial growth and physiology (Zhang et al., 2019).
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 (1% 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 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-ΔΔCt 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-32P]-ATP. The EMSA was performed in a 10 μl reaction volume containing binding buffer (1 mM MgCl2, 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 32P-labeled end were generated by

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**Table 1. Oligonucleotide primers used in this study**

| Target                      | Primers (forward/reverse, 5’-3’)                                      |
|-----------------------------|-----------------------------------------------------------------------|
| **qPCR**                    |                                                                         |
| flgM (VP0771)               | ATTCAAGTGCGACATCAAG/GCGAGAAGCTGCCATATC                                |
| flgA (VP0772)               | ATTGCGCCTGAACACTG/GATCGGTACTGAGAGGACAC                                |
| flgB (VP0775)               | ACAAGGCACTAGGCA/TGACCATCTGTGCTAAG                                     |
| flgK (VP0785)               | GCCGTCACTACTG/TGAGGAGACGAGTTGTC                                       |
| **LacZ fusion**             |                                                                         |
| flgM (VP0771)               | GCGCTCTAGATGTCCCTCTGTTGCTC/GCGCGAATTCTGACTGAAATGTTAC                 |
| flgA (VP0772)               | GCGCGTCGACTCTTGCTGTTGACTG/GCGCGAATTCTGACTGAAATGTTAC                 |
| flgB (VP0775)               | GCGCGTCGACTCTTGCTGTTGACTG/GCGCGAATTCTGACTGAAATGTTAC                 |
| flgK (VP0785)               | GCGCGTCGACTCTTGCTGTTGACTG/GCGCGAATTCTGACTGAAATGTTAC                 |
| **Protein expression**      |                                                                         |
| opaR                        | AGCCGCGAGCTTGAAGAGGAAGGCAGATTGTA/GAGCGGAATCTGGTTGCTGGAAGGACAGGTTGAG  |
| **EMSA**                    |                                                                         |
| flgM (VP0771)               | CGTAGCGTCGGTCTCTGTTGCTGTTGCTG/GCGCGAATTCTGACTGAAATGTTAC              |
| flgA (VP0772)               | TTGCCAGGTGGTCTAATTACATC/GCAGATGGATTATTGACTGACTG                      |
| flgB (VP0775)               | AATCCCGGTCTCAGGATATGCTTCATG/GCGCGAATTCTGACTGAAATGTTAC               |
| flgK (VP0785)               | AACCATCTTGCTTCTG/GCGCGAATTCTGACTGAAATGTTAC                          |
| **DNase I footprinting**    |                                                                         |
| flgM (VP0771)               | CGTAGCGTCGGTCTCTGTTGCTGTTGCTG/GCGCGAATTCTGACTGAAATGTTAC              |
| flgA (VP0772)               | TTGCCAGGTGGTCTAATTACATC/GCAGATGGATTATTGACTGACTG                      |
| flgB (VP0775)               | AATCCCGGTCTCAGGATATGCTTCATG/GCGCGAATTCTGACTGAAATGTTAC               |
| flgK (VP0785)               | AACCATCTTGCTTCTG/GCGCGAATTCTGACTGAAATGTTAC                          |
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 Ca²⁺/Mg²⁺ solution (5 mM CaCl₂ and 10 mM MgCl₂) 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.

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 OD₆₀₀ values of each strain were monitored at 1-h intervals to create growth curves. As shown in Fig. 2, indistinguishable growth rates were ob-
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, containing at least 11 operons: flgAMN, flgMN, cheVR, flgBCDEFGHIJ, flgKL-flaC, flgKL, flaDE, flaAGHIJK, flaKLM, flgEFGHIJ-KLMNOPQR-flhB, and flhAFG-fltA-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 flgB 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...
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 Δ*toxR* strain is dramatically decreased relative to WT (Chen et al., 2018). Swimming capacity is also significantly inhibited in the Δ*oxyR* 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 Vcd1 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 *flgBCD-EFGHIJ* within the Pof gene loci to repress their transcription, whereas it negatively regulated the transcription of *flgKL*, which regulates the swimming motility 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. tubiashi*, 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 activates 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. Downregulation 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 *flgBCD-EFGHIJ* 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^70^-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^54^-dependent promoter was predicted for *flgAMN* using the online Softberry tool (http://linux1.softberry.com/berry.phtml) (Fig. 5A). OpaR binding sites for *flgAMN* or *flgMN* were 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 *flgBCD-EFGHIJ* that initiate its transcription (Fig. 5C) (Kim and McCarter, 2000). The two OpaR binding sites for *flgBCD-EFGHIJ* entirely overlapped each of the promoters (Fig. 5C). Thus, OpaR repressed the transcription of *flgBCD-EFGHIJ*, 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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