

## Role of Dual Flagella in the Pathogenesis of *Vibrio parahaemolyticus*

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*Vibrio parahaemolyticus* possesses two flagella systems: polar and lateral flagella for swimming in liquid and swarming on solid surfaces or in viscous environments. To elucidate the pathogenic role of these dual flagella systems, we constructed single- and double-deletion mutants of the *lafA* and *flhAB* flagellum genes and investigated their biofilm formation, cell adhesion, and colonization of the small intestine of suckling mice. The double-mutant strain was more impaired in biofilm formation than either of the single-mutant strains. In addition, the *lafA*, *flhAB*, and double-mutant strains showed 40%, 45%, and 60%, respectively, lower adherence to HeLa cells than the wild-type strain. Moreover, the *lafA*, *flhAB*, and double-mutant strains exhibited 49%, 5.6 and 6.7 times, respectively, lower colonization in a competition assay than the wild-type strain. These findings indicated that polar flagella were more important than lateral flagella for the pathogenesis of *V. parahaemolyticus*.

Key words: *Vibrio parahaemolyticus*, Dual flagella, Biofilm formation, Adhesion, Competition

### Introduction

To adapt and survive under changing environmental conditions, bacteria can modulate their gene expression and direct their movements by various processes such as flagella-mediated chemotaxis. *Vibrio parahaemolyticus* is a Gram-negative bacterium that possesses two types of flagella on swimmer and swarmer cells, namely, polar and lateral flagella, which are used to swim in liquids and swarm on solid surfaces or in viscous environments, respectively (McCarter, 2004). Swimmer cells are rod-shaped, about 2 µm long, and have a single polar flagellum, while swarmer cells are multinucleate, more than 30 µm long, and have many lateral flagella (Shinoda et al., 1974; McCarter and Silverman, 1990). Polar flagella are formed constitutively, whereas lateral flagella are induced under conditions that disable the polar flagella, such as on solid surfaces, in viscous environments (McCarter et al., 1988), and in the presence of phenamil, a potent inhibitor of sodium ion channels (Klose and Mekalanos, 1998) that disrupt the sodium-motive force that powers flagella (Atsumi et al., 1992). Furthermore, the swimmer and swarmer cell types can reversibly

differentiate in different environments. For example, the synthesis of lateral flagella ceases when the environment changes from solid to liquid (Stewart and McCarter, 2003). More generally, the lateral flagella of *V. parahaemolyticus* form in response to changes in environmental factors, such as pH, temperature, and NaCl concentration (Belas and Colwell, 1982).

In *V. parahaemolyticus*, 60 polar flagellum genes are located exclusively on the large chromosome, whereas 38 lateral flagellum genes are found in two regions of the small chromosome (Makino et al., 2003; Stewart and McCarter, 2003). In addition, several regulatory genes, such as the *scrABC* operon, control the formation of lateral flagella (Boles and McCarter, 2002). For example, *LafK* and *FliA<sub>L</sub>* control the expression of early and late genes of the lateral flagellum of *V. parahaemolyticus* (Stewart and McCarter, 2003).

Recently, we reported that *VpaH*, which encodes a novel H-NS-like protein, regulates the formation of lateral flagella in *V. parahaemolyticus* TH3996, which is a strain that expresses TRH, a major virulence protein (Park et al., 2005). In addition, *LonS*, *OpaR*, and *SwrT* are involved in the formation of lateral flagella (Stewart et al., 1997; Enos-Berlage et al., 2005; Jaques and McCarter, 2006). Flagella

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play an important role in adhesion to substrates, biofilm formation, and virulence in pathogenic bacteria (Kawagishi et al., 1996; Ottemann and Miller, 1997). For example, the lateral flagella of *V. parahaemolyticus* are adapted for colonization on solid or mucosal surfaces and may also bind chitin (Belas and Colwell, 1982). In addition, its lateral flagella are involved in the formation of biofilm (Park et al., 2005), which is a matrix of bacterial communities on surfaces (Davey and O'toole, 2000; O'Toole et al., 2000). In *V. parahaemolyticus*, biofilm formation is mediated by many factors, including extracellular polysaccharides, mannose-sensitive hemagglutinin (MSHA) type 4 pili, chitin-regulated pili (ChiRP), and OpaR (Enos-Berlage et al., 2005; Shime-Hattori et al., 2006; Yildiz and Visick, 2009). However, the relationship between flagella proteins and biofilm formation is not clear and little is known about the pathogenic role of either polar or lateral flagella in *V. parahaemolyticus*.

As a result, we examined the phenotypic effect of single- and double-deletion mutants of the *lafA* and *flhAB* flagellum genes in this bacterium. These mutant strains exhibited significantly less biofilm formation, cell adhesion, and colonization of the small intestine of suckling mice compared to the wild-type.

## Materials and Methods

### Bacterial strains, plasmids, medium, and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *V. parahaemolyticus* TH3996 was used as the parental strain (Park et al., 2000), while *Escherichia coli* DH5 $\alpha$  and SM10 $\lambda$ pir (Miller and Mekalanos, 1988) were used for the general manipulation of plasmids and plasmid

mobilization. *E. coli* and *V. parahaemolyticus* were cultured in Luria-Bertani (LB) medium or LB broth with 3% NaCl at 35°C or 28°C, respectively. Thiosulfate-citrate-bile salts-sucrose agar (TCBS) agar (Nissui, Tokyo, Japan) and LB broth with 3% NaCl were used for screening mutant strains and biofilm formation assays. When necessary, the media were supplemented with antibiotics: ampicillin (25 or 100  $\mu$ g/mL), gentamicin (10  $\mu$ g/mL), and chloramphenicol (5  $\mu$ g/mL).

### Motility assay

For motility assays, we inoculated semisolid tryptone swarm plates containing 1% Bacto-tryptone, 2% NaCl, and 0.3% Bacto™ agar (Difco, Detroit, MI, USA) with 2  $\mu$ L of an overnight culture of bacteria normalized to an optical density of 1.0 at 600 nm (OD<sub>600</sub>). These plates were incubated at 28°C for 12 h, and then the motility of bacteria was assessed qualitatively by examining the circular swarms formed by the growing bacterial cells.

### Construction of deletion mutants

The *lafA* (VPA1548, lateral flagellin gene), *flhAB* (VP2235 and VP2236, polar flagella assembly genes), and  $\beta$ -lactamase (VPA0477) deletion mutants of *V. parahaemolyticus* TH3996 were constructed using overlap extension-PCR, as described previously (Park et al., 2005). To construct the *lafA* mutant, the primers were 1548-1 (5'-GGATCCCTTGTAACCTCAAAC-3'), 1548-2 (5'-TCAAAACCACCACCATCAGCGTCCATTGCT-3'), 1548-3 (5'-AGCAATGGACGCTGATGGTGGTGGTTTTGA-3'), and 1548-4 (5'-GGATCCATGATACGGCCTTTA-3'). To construct the *flhAB* mutant, the primers were 2235-1 (5'-GGATCCAACCTTAGCATGGATG-3'), 2235-2 (5'-AACGTTTCAGCGCC AAGTTTTCTGCTTATAA-3'), 2235-3 (5'-TTATAAGCAGAAAACCTTGGCGCTGACGTT-3'), and 2235-4 (5'-CTGCAGAAATCTGC

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>hsdR recA lacZYA <math>\pi</math>80 lacZ<math>\Delta</math>M15</i>	Laboratory collection
SM10 $\lambda$ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu<math>\lambda</math>pir R6K</i>	Miller and Mekalanos, 1988
<i>Vibrio parahaemolyticus</i>		
TH3996	Clinical isolate, <i>tdh</i> <sup>-</sup> , <i>trh</i> <sup>+</sup> , <i>ure</i> <sup>+</sup>	Park et al., 2000
3996-F1	TH3996, <i>lafA</i> deletion mutant	This study
3996-F2	TH3996, <i>flhAB</i> deletion mutant	This study
3996-F3	3996-F1, <i>flhAB</i> deletion mutant	This study
3996-F4	TH3996, $\beta$ -lactamase deletion mutant	This study
Plasmids		
pT7Blue T-vector	Multicopy (ColE1 <i>ori</i> ) TA cloning vector, Amp <sup>r</sup>	Novagen, Inc
pYAK1	Suicide vector, R6K <i>ori</i> , <i>sacB</i> , Cm <sup>r</sup>	Park et al., 2005

CTCTTTG-3'). To construct the  $\beta$ -lactamase mutant, the primers were 0477-1 (5'-CAAACCTGGATCCATTGCGCA-3'), 0477-2 (5'-TAGGGCGTTATCTTCTTTGCCGCTGTCCAT-3'), 0477-3 (5'-ATGGA CAGCGGCAAAGAAGATAACGCCCTA-3'), and 0477-4 (5'-CATTTGAGACTGCAGGTAAGA-3').

The final PCR products were cloned into the pT7Blue T-Vector (Novagen, Madison, WI, USA) and then restriction-digested. Restriction fragments were subsequently cloned into the pYAK1 suicide vector (Park et al., 2005). After introducing the resulting plasmids into *E. coli* SM10 $\lambda$ pir (Miller and Mekalanos, 1988), the transformed bacteria were conjugated with *V. parahaemolyticus* TH3996. Finally, the deletion mutants were screened on TCBS agar plates containing 5  $\mu$ g/mL chloramphenicol and then selected on LB plates supplemented with 10% sucrose.

#### Western blot analysis

Whole-cell lysates of bacteria ( $10^6$  CFU) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Afterward, the proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 and then probed with anti-flagellin antibody (1:3,000 dilution) for 30 min at room temperature. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:5,000 dilution) for 30 min at room temperature (Amersham, Piscataway, NJ, USA). Finally, the blots were visualized by using the enhanced chemiluminescence Western blotting kit (Amersham).

#### Adherence assay

HeLa cells ( $2 \times 10^5$ ) were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) in 12-well plates in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C overnight. In addition, overnight cultures of *V. parahaemolyticus* strains were diluted 1:100 in fresh LB broth containing 3% NaCl and grown at 37°C to the late log phase. Subsequently, the cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS, pH 7.2) and then used to infect HeLa cells at a multiplicity of infection of 10. After 1.5 h, non-adherent bacteria were removed by washing the plates with PBS. The adherent bacteria were then recovered by treatment with 0.2% Triton X-100 (Sigma) for 5 min at room temperature and then plated on LB agar containing

3% NaCl. The percentage adherence was calculated by dividing the adherence of the tested strains by that of the wild-type strain.

#### Biofilm formation assay

Overnight cultures of bacteria were inoculated at a 1:100 dilution into LB broth supplemented with 3% NaCl in borosilicate glass tubes and incubated for 24 h at 28°C. After rinsing the tubes with distilled water to remove non-adherent cells, they were filled with 1.0 mL of 0.2% crystal violet solution (Junsei Chemical Co., Ltd., Tokyo, Japan), incubated for 15 min, and then rinsed vigorously with distilled water. Subsequently, the dye was removed from the cells with 1.0 mL of dimethyl sulfoxide, and biofilm formation was quantified by measuring the optical density of this solution at 570 nm. All assays were performed in triplicate.

#### Suckling mouse colonization assay

We used the suckling mouse colonization assay of Gardel and Mekalanos (1996) with slight modifications. Briefly, 1:1 mixtures of mutant and parental strains of *V. parahaemolyticus* TH3996 (about  $3 \times 10^5$  total cells) were administered intragastrically to CD-1 suckling mice (5-6 days old). After 24 h, the small intestine was homogenized, and then adequately diluted samples were plated on TCBS agar with or without 25  $\mu$ g/mL ampicillin. The competitive index was calculated as the ratio of mutant/parental strains that were recovered from the small intestine. The mean competitive index was the average of four assays, each with at least five mice.

## Results

#### Construction of flagella gene deletion mutant strains

The *lafA*, *flhAB*, and double-deletion mutant strains were confirmed by PCR and Southern blotting, and their *in vitro* growth curves were indistinguishable from that of the wild-type strain (data not shown). Furthermore, Western blotting showed that polar flagellin was expressed in the wild-type strain and *lafA* deletion mutant strain but not in the *flhAB* deletion mutant or double-mutant strains (Fig. 1). Similarly, lateral flagellin was expressed in the wild-type strain and *flhAB* deletion mutant strain but not in the *lafA* deletion mutant or double-mutant strains (Fig. 1). These results indicate successful construction of three types of deletion mutant strains.

#### Motility of flagella gene deletion mutant strains

The swarming motility of the *lafA* and *flhAB* single deletion mutant strains was significantly lower than

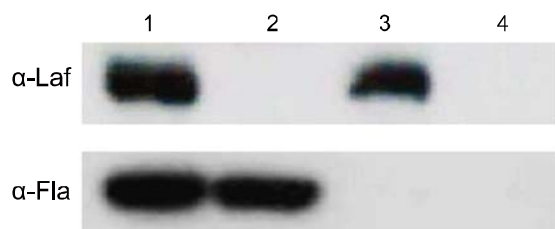


Fig. 1. Western blot analysis of whole-cell lysates of wild-type and flagella gene mutant strains of *Vibrio parahaemolyticus*. Lane 1, wild-type strain; lane 2, *lafA* deletion mutant strain; lane 3, *flhAB* deletion mutant strain; lane 4, *lafA/flhAB* double mutant strain.

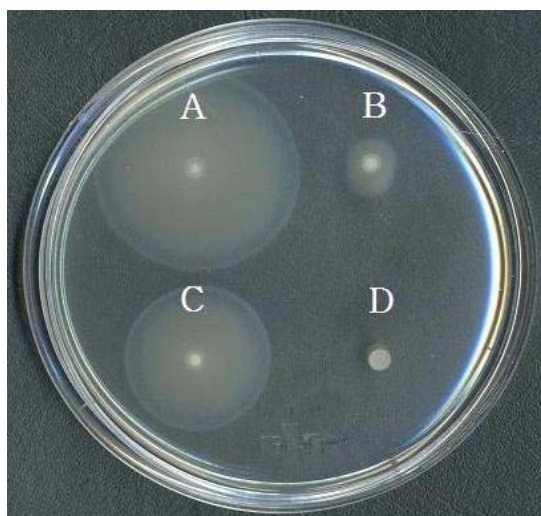


Fig. 2. Motility assay of wild-type and flagella gene deletion mutant strains of *Vibrio parahaemolyticus*. A, wild-type strain; B, *lafA* deletion mutant strain; C, *flhAB* deletion mutant strain; D, *lafA/flhAB* double mutant strain.

that of the wild-type (Fig. 2A-2C). The small amount of motility in the *lafA* deletion mutant was most likely due to the residual activity of polar flagella. The double-mutant strain was nonmotile (Fig. 2D). Consequently, these results coincided with the Western blot analysis shown in Fig. 1.

#### Biofilm formation and cell adhesion of flagella gene deletion mutant strains

The wild-type strain rapidly formed biofilm until it peaked during the stationary growth phase and then slowly declined (Fig. 3). In contrast, biofilm formation was impaired in all three flagellum gene deletion mutants. Specifically, the *lafA* deletion mutant exhibited less biofilm formation than the wild-type strain. Similarly, the *flhAB* deletion mutant adhered to host cells and formed biofilm more slowly than either the *lafA* deletion mutant or the wild-type

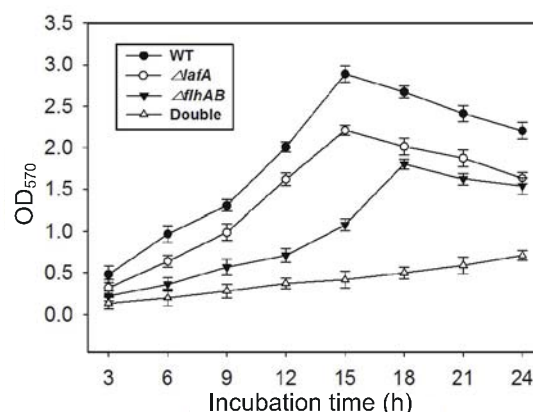


Fig. 3. Ability of wild-type (WT) and flagella gene deletion mutant strains of *Vibrio parahaemolyticus* to form biofilms. Results are expressed as the mean of three independent experiments. Error bars represent SDs.

strain. Not surprisingly, the double mutant exhibited the most severely impaired biofilm formation.

In the adherence assays, the *lafA*, *flhAB*, and double-mutant strains showed 40%, 45%, and 60%, respectively, less adherence to HeLa cells than the wild-type strain (Fig. 4). Together, these results demonstrated that both polar and lateral flagella of *V. parahaemolyticus* are critical for biofilm formation and cell adhesion.

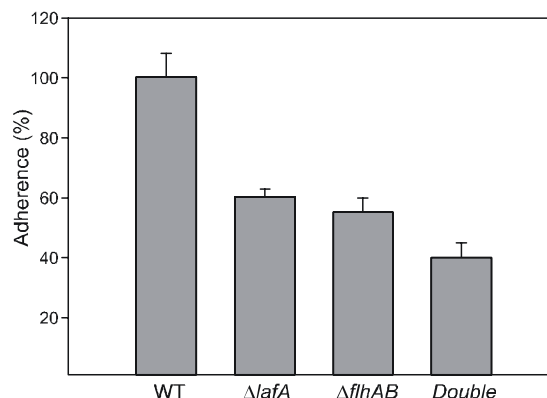


Fig. 4. Adherence of wild-type (WT) and flagella gene deletion mutant strains of *V. parahaemolyticus* to HeLa cells. Results are expressed as the ratio of the adherence of the tested strains to that of the wild-type strain. Error bars represent SDs.

#### Colonization of flagella gene deletion mutant strains in suckling mice

The *lafA*, *flhAB*, and double-mutant strains exhibited 49%, and 5.6 and 6.7 times lower colonization, respectively, in the competition assay than the

wild-type (Table 2). These results indicated that the ability of *V. parahaemolyticus* to colonize the small intestine of suckling mice depends more on polar than lateral flagella.

Table 2. Infant mouse colonization assay of dual flagella-related gene deletion mutants

Strains	Relevant genotype	Competitive index*
3996-F1	$\Delta lafA$	$0.77 \pm 0.14$ ( $n=16$ )
3996-F2	$\Delta flhAB$	$0.18 \pm 0.04$ ( $n=15$ )
3996-F3	$\Delta lafA, \Delta flhAB$	$0.15 \pm 0.05$ ( $n=18$ )

\*The competition index is given as the ratio of output mutant to wild type (recovered from the intestine) divided by the ratio of input mutant to wild type (inoculated into a mouse). Thus, if a mutant has no colonization defect, we expect a competitive index close to 1.

## Discussion

Although many studies have characterized flagellum genes and proteins of the dual flagella system of *V. parahaemolyticus*, few have investigated their pathogenic role. Moreover, the role of the polar flagella is less clear than that of the lateral flagella. In this study, we constructed three flagellum gene deletion mutant strains of *V. parahaemolyticus* and determined their effects on biofilm formation, cell adhesion, and colonization of the small intestine of suckling mice. Our results were consistent with previous studies, which demonstrated that lateral and polar flagella are associated with cell adhesion and colonization (Belas and Colwell, 1982) as well as biofilm formation (Enos-Berlage et al., 2005; Park et al., 2005). Similar studies on two other *Vibrio* spp., namely *V. vulnificus* and *V. cholerae*, also suggested that the loss of flagellum genes usually results in decreased attachment, biofilm formation, and virulence (Watnick et al., 2001; Lee et al., 2004). However, unlike *V. parahaemolyticus*, neither *V. vulnificus* nor *V. cholerae* possesses lateral flagella. In the adherence and biofilm formation assays in this study, the flagellum gene deletion mutant strains of *V. parahaemolyticus* exhibited significantly less adherence to HeLa cells and biofilm formation than the wild-type. Therefore, our data suggest that *lafA* and *flhAB* are positively regulated with other genes that are related to cell adhesion and biofilm formation, such as those that control the expression of pili, outer membrane proteins, capsular polysaccharides, and exopolysaccharides. Furthermore, in the competition assay in suckling mice, both the *flhAB* and double-mutant strains showed a severe colonization defect,

however, the *lafA* mutant strain did not exhibit significantly reduced colonization. These results suggest that polar flagella are more important than lateral flagella for colonization. Since lateral flagella of wild-type *V. parahaemolyticus* are extremely unlikely to form in liquid environments, we hypothesize that they cannot be formed in the small intestine of mice. Therefore, further study is needed to elucidate the expression pattern of lateral flagella in *V. parahaemolyticus*.

In conclusion, this study showed that both the lateral and polar flagella of *V. parahaemolyticus* are involved in biofilm formation, cell adhesion, and colonization of the small intestine in suckling mice. This study has important implications for further research into the pathogenesis of *V. parahaemolyticus*.

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