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Roles of Flagellar Hook-Associated Proteins in *Vibrio vulnificus* Motility and Virulence

Soo Young Kim[1,3], Hye Young Hong[3], Joon Haeng Rhee[1,2,3*] and Sun Sik Chung[2]

[1]Clinical Vaccine R&D Center, Chonnam National University Hwasun Hospital, Jeonnam 519-809,
[2]Research Institute of Vibrio Infections and Genome Research Center for Enteropathogenic Bacteria,
[3]National Research Laboratory of Molecular Microbial Pathogenesis and Department of Microbiology,
Chonnam National University Medical School, Gwangju 501-746, South Republic of Korea

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The bacterial flagellar structure can be divided into the basal body, the hook and the filament. Three minor components called hook associated proteins (HAP1, HAP2 and HAP3) form a junction between the hook and the filament (HAP1 and HAP3) and a capping structure at the distal end of flagellar filament (HAP2). *Vibrio vulnificus* is a halophilic pathogenic bacterium that is locomotive by means of a polar flagellum. From a *V. vulnificus* genome sequencing project, we obtained sequences of *V. vulnificus flgK* (Vv-*flgK*), *flgL* (Vv-*flgL*), and *flaH* (Vv-*flaH*) genes that encode HAP1, HAP3, and HAP2, respectively. To investigate roles of the HAP proteins, deletion mutants of the Vv-*flgK*, Vv-*flgL* and Vv-*flaH* were constructed. Electron microscopic analysis showed that the Vv-*flgK* or Vv-*flgL* mutant did not produce an intact polar flagellum while the Vv-*flaH* mutant produced a fragile flagellar structure. Western blot analysis against a major polar flagellin proposed that the null HAP1 and HAP3 mutations resulted in a failure of normal flagellar assembly since flagellins produced by the mutants were secreted out in the culture supernatants without long flagellar filaments. Motility was completely abolished by a single mutation in HAP1 or HAP3, and the HAP2 mutant showed a decreased motility. Also each of the mutants showed an impaired cytotoxicity and adherence to HeLa cell compared with the isogenic wild type strain. LD_{50} increased by 10- and 11-fold in the *V. vulnificus* HAP3 and HAP2 mutant, respectively. These results suggest that the HAP proteins play important roles in polar flagellation and the virulence of *V. vulnificus*.

Key Words: *Vibrio vulnificus*, Flagella, Hook associated proteins, Motility, Virulence

INTRODUCTION

Vibrio vulnificus is a halophilic estuarine bacterium that causes fatal septicemia and necrotizing wound infections. Primary septicemia occurs following ingestion of raw seafood contaminated with *V. vulnificus*. *V. vulnificus* preferentially affects persons with underlying hepatic diseases, heavy alcohol drinking habits, and other immunocompromised conditions. Primary septicemia shows a rapidly progressing and fulminant course, which results in a high mortality rate over 50% despite of aggressive antimicrobial and supportive shock therapies (26,27,31). Several virulence factors are reported to date to play important roles in the pathogenesis of *V. vulnificus* septicemia: RTX toxin (18), hemolysin (4, 25,32), protease (19), siderophores (20) and capsular polysaccharide (5) etc. In addition to these factors, adhesion and invasion to host cell could be another virulence determinants of *V. vulnificus*. *V. vulnificus* that was ingested orally and

*Corresponding author: Joon Haeng Rhee. Department of Microbiology, Chonnam National University Medical School, 5-1 Hak-dong, Dong-ku, Gwangju 501-746, South Republic of Korea.
Phone: +82-62-220-4136, Fax: +82-62-228-7294
e-mail: jhrhee@chonnam.ac.kr
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Table 1. Bacterial strains used in this study

Strain	Characteristics	Source or reference
V. vulnificus		
CMM2301	CMCP6 with Vv-*flgK* deletion mutation	This study
CMM2302	CMCP6 with Vv-*flgL* deletion mutation	This study
CMM2305	CMCP6 with Vv-*flaH* deletion mutation	This study
CMM2303	CMM2301 harboring pCMM410	This study
CMM2304	CMM2302 harboring pCMM410	This study
CMM2306	CMM2305 harboring pCMM413	This study
E. coli		
SY327 *λ pir*	Δ(*lac pro*) *argE*(Am) *rif nalA recA*56 *pir* lysogen; host for π-requiring plasmids	(23)
SM10 *λ pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc^{R}::Mu *λ pir* lysogen, oriT of RP4, Km^{R}; Conjugal donor	(23)
DH5α	F^{-} *recA*1; restriction negative	Laboratory collection

survived in acidic gastric environment reaches to small intestine. In the small intestine, the pathogen adheres to intestinal epithelium, invades into lamina propria and eventually blood stream, and multiplicate in blood to establish septicemia. Therefore, the adhesion and invasion steps might play an important role in the very early stage of the infection. In addition to their primary role in cellular locomotion, flagella were reported to play an important role in adherence of enteropathogenic *Escherichia coli* to epithelial cells (3).

The bacterial flagellum is an organelle for motility. The flagellum can be divided into three major portions, the basal body, the hook and the filament. The basal body is lodged in the cell membrane and acts as a rotary motor to drive the helical filament rotation that results in bacterial propulsion. The filament is a helical propeller composed of flagellin subunits and the hook is a universal joint that transmits the torque to the helical filament. There are other accessory components in the flagellar structure which they are called hook associated proteins, HAP1, HAP2 and HAP3 (2,8,9). HAP1 and HAP3 are located between the hook and the filament to form the junction between these two filamentous structures (10,12). HAP2 forms the capping structure at the distal end of flagellum, where the cap promotes incorporation of flagellin monomers into the filament structure for efficient flagellar growth (11,33).

In the present study, we identified *flgK* (Vv-*flgK*), *flgL* (Vv-*flgL*) and *flaH* (Vv-*flaH*) genes of *V. vulnificus* that encode HAP1, HAP3 and HAP2, respectively. To understand a relationship between the HAPs and flagellation/virulence of *V. vulnificus*, we evaluated roles of HAPs in motility, cytotoxicity/adherence to HeLa cell, and lethality to mice by using in-frame deletion mutants of the Vv-*flgK*, Vv-*flgL* and Vv-*flaH*.

MATERIALS AND METHODS

1. Bacterial strains, plasmids, media and reagents

Strains and plasmids used in this study are listed in the Tables 1 and 2, respectively. *V. vulnificus* CMCP6, a highly virulent clinical isolate from a Korean septicemic patient, was used. *E. coli* and *V. vulnificus* were routinely grown in Luria-Bertani (LB) medium and 2.5% NaCl heart infusion (HI) medium, respectively. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin 100 μg/ml, chloramphenicol 30 μg/ml, kanamycin 100 μg/ml and tetracycline 12.5 μg/ml; for *V. vulnificus*, ampicillin 20 μg/ml, chloramphenicol 2 μg/ml and tetracycline 2 μg/ml. Bacteria were grown by shaking at 200 rpm and 37℃ overnight in appropriate media.

2. Construction of deletion mutants of Vv-*flgK*, Vv-*flgL* and Vv-*flaH* genes

To investigate the function of Vv-FlgK, Vv-FlgL and Vv-FlaH, in-frame deletion mutants of Vv-*flgK*, Vv-*flgL*

Table 2. Plasmids used in this study

Plasmid	Characteristics	Source or reference
pCR2.1	PCR TOPO TA cloning vector	Invitrogen
pDM4	Suicide vector with R6K *ori* (requires π) *sacB* and Cm^R	(24)
pLAFR3II	pLAFR3 with *bla* instead of cos site	(15)
pRK2013	IncP, Km^R, Tra $Rk2^+$ *repRK2 repE1*	(1)
pCMM405	pCR2.1 having a in-frame deleted 1.5-kb Δ*flak* fragment	This study
pCMM406	pCR2.1 having a in-frame deleted 1.7-kb Δ*flaL* fragment	This study
pCMM411	pCR2.1 having a in-frame deleted 1.4-kb Δ*flaH* fragment	This study
pCMM407	*Sac*I-*Xba*I fragment containing a in-frame deleted Δ*flak* of pCMM405 cloned into pDM4	This study
pCMM408	*Sac*I-*Xba*I fragment containing a in-frame deleted Δ*flaL* of pCMM406 cloned into pDM4	This study
pCMM412	*Xba*I-*Hin*dIII fragment containing a in-frame deleted Δ*flaH* of pCMM411 cloned into pDM4	This study
pCMM409	3.9-kb PCR fragment containing Vv-*flgK* and Vv-*flgL* cloned into pCR2.1	This study
pCMM410	3.4-kb fragment containing Vv-*flgK* and Vv-*flgL* of pCMM409 cloned into pLAFR3II	This study
pCMM413	3.4-kb PCR fragment containing Vv-*flaH* cloned into pCR2.1	This study
pCMM414	3.47-kb fragment containing Vv-*flaH* of pCMM413 cloned into pLAFR3II	This study

and Vv-*flaH* were constructed with *V. vulnificus* CMCP6. We designed two sets of primers amplifying the DNA fragments containing upstream and downstream of Vv-*flgK*, Vv-*flgL* or Vv-*flaH*. Sequences of the primer sets are listed in the Table 3. PCR was performed with the Mastercycler (Eppendorf, Hamburg, Germany). The amplified DNA fragments were cloned into pCR2.1 vector using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). The in-frame deleted fragments of each gene were created by a ligation of each downstream and upstream PCR fragments yielding pCMM405, pCMM406 or pCMM411 for ΔVv-*flgK*, ΔVv-*flgL* or ΔVv-*flaH*, respectively.

In-frame deletion of Vv-*flgK*, Vv-*flgL* or Vv-*flaH* was constructed by using the counter selection strategy employing a suicide vector pDM4 (24,29). A *Sac*I-*Xba*I DNA fragment containing deletion of Vv-*flgK* or Vv-*flgL* and a *Hin*dIII-*Xho*I fragment containing deletion of Vv-*flaH* were isolated from pCMM405 or pCMM406 and pCMM411, respectively, and ligated to the suicide vector pDM4. The resulting DNA ligate was transformed into *E. coli* SY327 λ *pir* by electroporation. Chloramphenicol resistance (Cm^R) transformants were selected and an appropriate plasmid was analyzed by restriction mapping to identify a plasmid carrying correct insert. The resulting plasmid pCMM407, pCMM408 or pCMM412 (Table 2) was transformed into *E. coli* SM10 λ *pir*, which provides trans-acting conjugative function for the plasmid (30). The plasmid was transferred to the wild type *V. vulnificus* by conjugation. Stable Cm^R transconjugants were selected on a thiosulfate-citrate-bile salt sucrose (TCBS)

Table 3. Primers used in this study

Primer	Sequence
flgK-3	5'-CATCAGCGGCAACCACACTGCC-3'
flgK-4	5'-GGTACCTAGAGGGAGCAGGTAAATGATTAGCC-3'
flgK-6	5'-AGTTGGCAATGCGTGAAGCGGG-3'
*flgK*7-1	5'-GGTACCCATAATGCCCCCAAAAACTCAATC-3'
flgL-1	5'-TCATTCATCGCCCCTTCGGCGG-3'
flgL-2	5'-GGATCCTAGTGAGCAAGAGCGAACCGCC-3'
*flgL*3-1	5'-GGATCCCATTTACCTGCTCCCTCTATCTCA-3'
*flgL*4-1	5'-CCGCATGGCAGCCGGATTTTCT-3'
flgKL-1	5'-GGATCCTACCGTCATCTCATTCAGAGGC-3'
flaH-1	5'-GTGCAATATCACCACCGTCATCCA-3'
flaH-2	5'-GGATCCTAGTAACGCATGACTCAACTTTCT-3'
flaH-3	5'-GGATCCCATCAAATCACCTCAATAACGAC-3'
flaH-4	5'-GACGGTTGCTGGCTCTCAAGAAGC-3'

agar plate containing Cm. Plating of the transconjugants on 2.5% NaCl HI agar plate containing 10% sucrose was performed to select clones that experienced the second homologous recombination events forcing excision of the vector sequence and leaving only mutated or wild type allele of Vv-*flgK*, Vv-*flgL*, or Vv-*flaH*. PCR was used to screen mutants with the appropriate allelic replacement.

3. Complementation

To restore the Vv-*flgK* and Vv-*flgL* wild type genes in the Vv-*flgK* and Vv-*flgL* mutant strains, we designed a set of primers (flgKL-1 and flgK-6) that could amplify a DNA fragment containing both Vv-*flgK* and Vv-*flgL* genes. For the Vv-*flaH* complementation, we used a set of primer flaH-1 and flaH-4 to be contained Vv-*flaH* ORF. The amplified DNA fragments were cloned into the pCR2.1 vector using the Original TA Cloning Kit (Invitrogen). The resulting plasmid was designated pCMM409 and pCMM413 for complementation of Vv-*flgK/L* and Vv-*flaH*, respectively. A 3,370-bp *Bam*HI fragment of pCMM409 and a 3,470-bp *Xba*I-*Hin*dIII fragment of pCMM413 were subcloned into the broad host range vector pLAFR3II (15) yielding pCMM410 and pCMM414, respectively. The pCMM410 was transferred into the Vv-*flgK* or Vv-*flgL* mutant strain, and pCMM414 plasmid was transferred into the Vv-*flaH* mutant by triparental mating using a conjugative helper plasmid, pRK2013 (1). The transconjugants were screened on TCBS agar plate containing appropriate antibiotics and confirmed by PCR.

4. Motility test

Motility assays were conducted on 0.3% agar-2.5% NaCl HI plates. One microliter of 10^9 CFU/ml bacterial suspension was inoculated on the motility assay agar and incubated at 37℃. Zones of migration were observed after 6 hours.

5. Western blotting

V. vulnificus CMCP6, CMM2301 and CMM2302 were grown overnight at 37℃ in 2.5% NaCl HI broth. The overnight culture was diluted by 200-fold by fresh medium and incubated by agitation at 200 rpm and 37℃. Bacterial cells were harvested by centrifugation. Both the bacterial pellet and supernatant were subjected to sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Constant numbers of cells (1×10^8 CFU) or equal volumes (20 μl) of the supernatant were mixed with sample loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodesyl sulfate, 0.1% bromophenol blue, 10% glycerol] and heated at 100℃ for 5 min. After separation by SDS-PAGE, the proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane. The antiserum used to probe the transferred proteins was raised in a New Zealand white rabbit by immunizing with GST-FlaB (14).

6. Adhesion assay

For the adhesion assay of the *V. vulnifucs* wild type and mutant strains to epithelial cells, HeLa cell line was used. HeLa cells were seeded into 4-well LabTec chamber slides (Nunc, Rochester, NY, USA) at a concentration of 1×10^5 cells/ml. Approximately 16 hours later, when the cells were subconfluent, medium was removed, and the cells were washed twice with pre-warmed serum-free DMEM. *V. vulnificus* cells at the exponential growth phase in 2.5% NaCl HI broth were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS), and resuspended in PBS to 1×10^9 CFU/ml. The HeLa cells were infected at the multiplicity of infection (MOI) of 250. After 30 min of incubation at 37℃, each well was washed three times with pre-warmed Hank's Balanced Salts Solution (Sigma-Aldrich Co., St. Louis, MO, USA) to remove non-adherent bacteria, and was stained with Giemsa solution (Merck, Darmstadt, Germany). Bacterial cells adhered to at least 30 HeLa cells were counted. Abilities of a bacterial strain to adhere to HeLa cells were compared as the average number of adhered bacteria per the cell.

7. Cytotoxicity assay

The CytoTox nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) was used to quantitate cytosolic lactate dehydrogenase (LDH) release as an indicator of cytotoxicity. HeLa cells were seeded in 24-well culture plates (Corning®, Corning, NY, USA) at a concentration of

1×10^5 cells/ml and cultured at 37℃ and 5% CO_2. After 24 hours, the cells were washed twice with 1 ml of pre-warmed serum-free DMEM. *V. vulnificus* cells at the exponential growth phase in 2.5% NaCl HI broth were harvested by centrifugation and washed three times with PBS, and resuspended in PBS to 1×10^9 CFU/ml. The HeLa cells were infected with the bacteria at the MOI of 100. After 90 min, the supernatants were harvested by centrifugation for 5 min at 13,000 rpm and 4℃. 30 μl of each supernatant was transferred to a 96-well plate and mixed with same volume of reconstituted LDH substrate mix. After 30 min incubation at room temperature in dark condition, 30 μl of stop solution was added to each well and the absorbance at 490 nm was measured. Complete cell lysate by 10% Triton X-100 and PBS served as the positive and negative control, respectively.

8. Electron microscopy

The bacteria strains were grown to mid-log phase in 2.5% NaCl HI broth without agitation. A formbar-coated grid was floated on a drop of the bacterial culture. The grids were floated onto a drop of 1% uranyl acetate. Bacterial cells were observed with H7600 electron microscope (Hitachi, Ibaraki, Japan).

9. LD_{50} determination

Intraperitoneal LD_{50} of the mutant and wild type strains were measured in randomly bred specific pathogen free (SPF) CD-1 mice (Daehan animal Co., Daejeon, Korea). Bacteria were grown in 2.5% NaCl HI broth for overnight at 37℃. Subsequently, 1 ml of the overnight culture was inoculated into 100 ml of the same fresh medium and grown at 37℃ and 200 rpm. After 4 hours, the bacterial cells were harvested by centrifugation, washed three times, resuspended, and diluted with PBS (pH 7.2). Eight-week-old female mice were challenged by intraperitoneal injection of 10-fold serial dilution of the bacterial suspensions. The infected mice were observed for 24 hours, and the LD_{50} was calculated by the method of Reed and Muench (28). All animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University.

RESULTS

1. Sequence analysis and organization of Vv-*flgK*, Vv-*flgL* and Vv-*flaH* genes

We have identified Vv-*flgK*, Vv-*flgL* and Vv-*flaH* encoding HAP1, HAP3 and HAP2, respectively, in the genome database of *V. vulnificus* CMCP6 (NCBI microbial genome database; http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html). There are two polar flagellar gene clusters in the *V. vulnificus* chromosome. Vv-*flgK/L* and Vv-*flaH* genes are located in different clusters with high sequence and organization similarities to those of *V. parahaemolyticus* (16, 21,22). Vv-*flgL* started at downstream of Vv-*flgK* with an intergenic space of 13-bp. A putative ribosomal binding site was observed at the intergenic sequence. Vv-*flgK* and Vv-

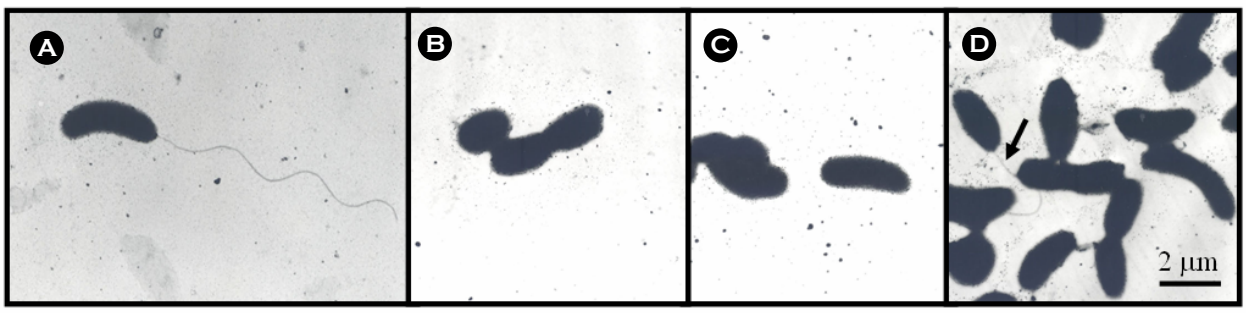

Figure 1. Electron microscopic photograph for showing effects of Vv-*flgK*, Vv-*flgL* and Vv-*flaH* mutations on *V. vulnificus* flagellation. Bacterial cells were grown in 2.5% NaCl HI and stained with 1% uranyl acetate. **A,** Wild type *V. vulnificus* CMCP6; **B,** CMM2301, a deletion mutant of Vv-*flgK*; **C,** CMM2302, a deletion mutant of Vv-*flgL*; **D,** CMM2305, a deletion mutant of Vv-*flaH*. The arrow indicates a truncated flagellum that was produced by the Vv-*flaH* mutant.

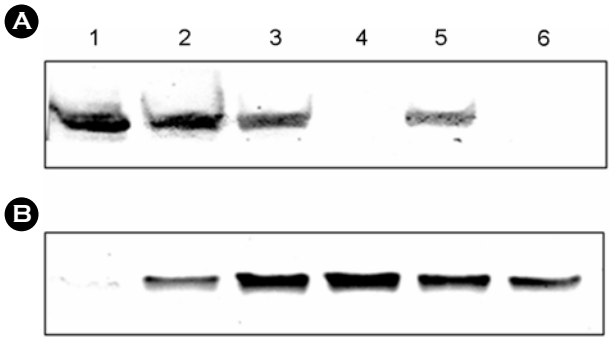

Figure 2. Western blot analysis showing effects of deletion mutation in the Vv-*flgK* or Vv-*flgL* on *V. vulnificus* flagellation. Cell pellets **(A)** containing equal numbers (1×10^8 CFU/ml) of the cells and supernatants **(B)** containing equal volumes (20 μl) were subjected to SDS-PAGE, and the proteins on the gels were electrophoretically transferred onto nitrocellulose membranes. Western blotting was carried out with anti-GST-FlaB serum. 1 and 2, Wild type *V. vulnificus* CMCP6, log and stationary phase; 3 and 4, CMM2301 (a deletion mutant of Vv-*flgK*), log and stationary phase; 5 and 6, CMM2302 (a deletion mutant of Vv-*flgL*), log and stationary phase.

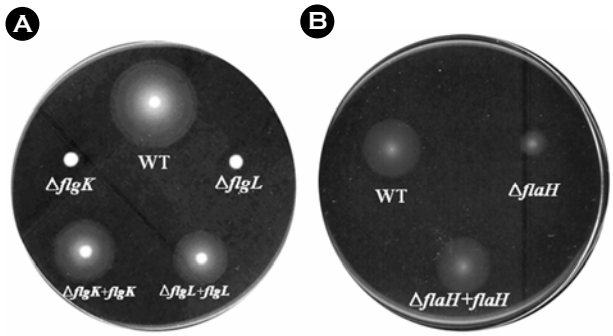

Figure 3. Effect of Vv-*flgK*, Vv-*flgL* and Vv-*flaH* mutations on motility. Both HAP1 and HAP3 mutants of *V. vulnificus* were nonmotile. HAP2 mutant showed a significant decrease in motility. WT, wild type; Δ*flgK*, CMM2301, a deletion mutant of Vv-*flgK*; Δ*flgL*, CMM2302, a deletion mutant of Vv-*flgL*; Δ*flaH*, CMM-2305, a deletion mutant of Vv-*flaH*. Δ*flgK* + *flgK*, Δ*flgL* + *lgL*, and Δ*flaH* + *flaH* shows Vv-*flgK*, Vv-*flgL*, and Vv-*flaH* mutant strains complemented by their wild type alleles.

flgL are supposed to comprise an operon. Deduced amino acid sequences of the Vv-FlgK, Vv-FlgL and Vv-FlaH showed 67%/70%, 76%/72% and 72%/62% identities with HAP1, HAP3 and HAP2 proteins of *V. parahaemolyticus* /*V. cholerae*, respectively.

2. Construction of Vv-*flgK*, Vv-*flgL* and Vv-*flaH* mutants

To analyze the effects of Vv-*flgK*, Vv-*flgL* and Vv-*flaH* null mutations on *V. vulnificus* flagellation and virulence, each deletion mutant strain was constructed by an allelic replacement using the positive selection vector pDM4. The resulting mutants were observed with an electron microscope. Both of the mutants Vv-*flgK* and Vv-*flgL* showed no flagellar filament whereas the wild type strain expressed an intact polar flagellum (Fig. 1). This result indicates that mutations in the Vv-*flgK* and Vv-*flgL* result in the defect of flagellation by preventing a normal HAP-mediated association of the flagellar hook with filament. In the Vv-*flaH* mutant, it has been shown that some bacteria had single truncated flagellum and others have no flgellum (Fig. 1). These results suggest that flagellar filaments were not properly assembled in HAP1 and HAP3 mutants, and HAP2 mutation resulted in production of a fragile flagellum.

The effect of the null HAP1 and HAP3 mutations on the flagella biogenesis was tested by western blot analysis of a major flagellin of *V. vulnificus*, FlaB (14). Exponential and stationary cultures of the wild type strain CMCP6, the Vv-*flgK* mutant CMM2301 and the Vv-*flgL* mutant CMM2302 were tested. Flagellins in the pellet and culture supernatant were analyzed by using anti-FlaB antiserum. The pellets of Vv-*flgK* and Vv-*flgL* mutants showed significantly decreased FlaB content with compared to that of the isogenic wild type strain during both exponential and stationary growth phases (Fig. 2A). On the other hand, the culture supernatants of Vv-*flgK* and Vv-*flgL* mutants contained more FlaB protein than that of the isogenic wild type (Fig. 2B). These results indicate that the mutations of Vv-*flgK* and Vv-*flgL* lead to a failure of the normal flagellar assembly so that the most flagellins produced by both mutants secret out in the supernatants.

3. Effect of HAP mutations on motility

Effects of HAP mutations on motility were tested using semisolid agar plate containing 0.3% agar (Fig. 3A and B). Motility was completely abolished even by single mutation in HAP1 or HAP3. The HAP2 mutant showed a significantly decreased motility. The motility defect of the mutants was complemented *in trans* by a wild type Vv-*flgK/L* or Vv-*flaH* alleles. This complete loss of motility in the HAP1

and HAP3 mutants could be attributed to the lack of flagellar filament assembly on the hook structure.

4. Effect of HAP mutations on cytotoxicity and adherence to HeLa cell

Virulent *V. vulnificus* strains are highly cytotoxic to wide spectrum of eukaryotic cells. Adhesion to epithelial cells is one of prerequisites for the cytotoxic mechanism of *V. vulnificus* (17). We tested effects of the HAP mutations on cytotoxicity and adherence of *V. vulnificus* to HeLa cell. The Vv-*flgK* and Vv-*flgL* mutants (CMM2301 and CMM-2302) were remarkably less cytotoxic to HeLa cells than the parent CMCP6 strain (Fig. 4A). The Vv-*flaH* mutant showed a significantly decrease in the cytotoxicity (Fig.

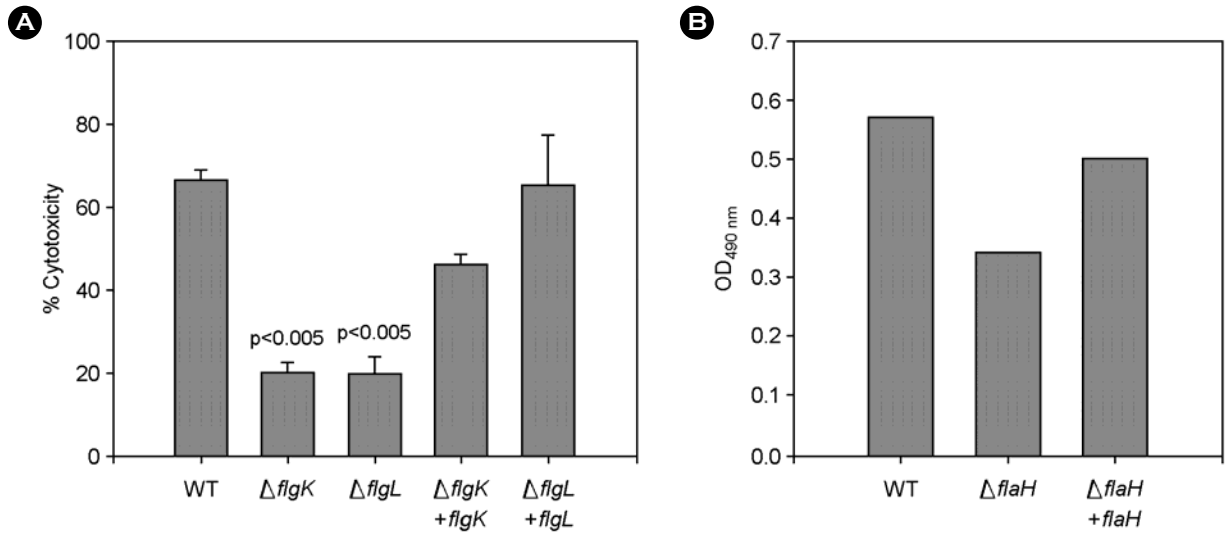

Figure 4. Effect of Vv-*flgK*, Vv-*flgL* **(A)** and Vv-*flaH* **(B)** mutations on the *V. vulnificus* cytotoxicity to HeLa cells. HeLa cells were infected at an MOI of 100:1 and LDH assays were carried out after 90 min incubation. The mean LDH released by the isogenic wild type strain *V. vulnificus* CMCP6 was set as 65%. The mean and the standard error of the mean were calculated from four or more separate experiments. Values represent the mean ± SEM. *p<0.05 versus the wild type strain.

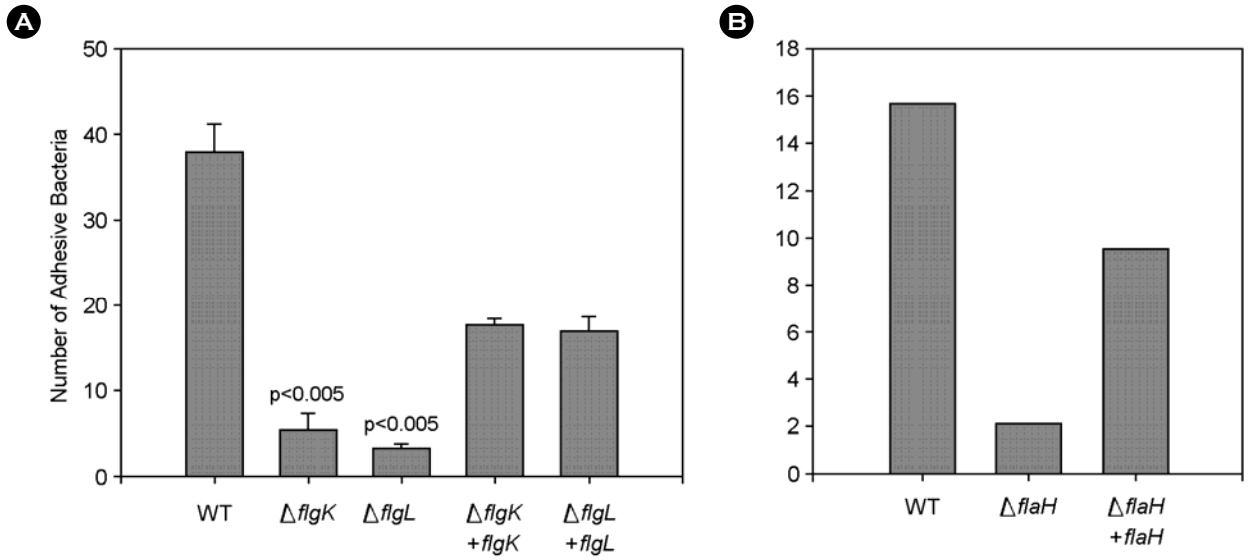

Figure 5. Effect of Vv-*flgK*, Vv-*flgL* **(A)** and Vv-*flaH* **(B)** mutations on the *V. vulnificus* adhesion to HeLa cells. HeLa cells were incubated with *V. vulnificus* strains at bacteria to cell ratio of 250:1 and incubated for 30 min. Cells were washed, fixed, stained with Giemsa, and observed under a microscopic at 1000×. Values of the mean and standard error of the mean were calculated by counting adhered bacteria to at least 30 HeLa cells. Values represent the mean ± SEM. *p<0.05 versus the wild type strain.

Table 4. Effects of Vv-*flgL* and Vv-*flaH* mutations on the LD_{50} of *V. vulnificus* to mice

Strain	LD_{50} (CFU)
CMCP6 (WT)	5.5×10^5
CMM2302 (Δ*flgL*)	5.5×10^6
CMM2305 (Δ*flaH)*	6.1×10^6

4B). The cytotoxicity defects were fully restored by an *in trans* complementation with plasmid encoding the wild allele. Adhesion of the mutants to HeLa cells significantly decreased compared with the isogenic wild type strain. The complemented strains showed restored adhesion to HeLa cell up to the wild type level (Fig. 5A and 5B).

5. Effect of HAP mutations on the lethality to mice

To address whether the HAPs plays an important role in *in vivo* polar flagellation and virulence of *V. vulnificus*, the Vv-*flgL* mutant CMM2302 and Vv-*flaH* mutant CMM-2305 were infected intraperitoneally to mice. LD_{50} of the mutants to CD-1 mice increased 10-fold and 11-fold for CMM2302 and 2305, respectively, in comparison with that of the wild type. LD_{50} of the wild type was 5.5×10^5 CFU, while those of the mutant were 5.5×10^6 CFU and 6.1×10^6 CFU (Table 4).

DISCUSSION

In the present study, we have identified and characterized the *V. vulnificus* Vv-*flgK*, Vv-*flgL* and Vv-*flaH* genes encoding HAP1, HAP3 and HAP2, respectively. A null mutation in the Vv-*flgK* or Vv-*flgL* resulted in a defect in polar flagellation abolishing motility completely and in a concomitant decrease in the cytotoxicity, adhesion and lethality to mice. A deletion mutation of Vv-*flaH* resulted in production of a fragile polar flagellum and a decrease in the cytotoxicity, adhesion and lethality to mice. This is the report showing that hook-associated proteins, HAP1, HAP3 and HAP2 which are auxiliary components of the flagellum, might play an important role in polar flagellation and virulence of *V. vulnificus*.

There are several reports showing different HAP mutant phenotypes in different bacteria. *S. typhimurium* mutants with defects in genes encoding HAP1, HAP2 or HAP3 were immotile and secreted unpolymerized filament protein into the growth medium (6,7,13). On the other hand, *V. parahaemolyticus* mutants defective for those genes showed different phenotypes in comparison with the *S. typhimurium* mutants (22). A mutant defective of the capping protein (HAP2) was motile but moved slowly in semisolid motility plates. The mutants with defects in the joining proteins (HAP1 and HAP3) were immotile but did not secrete unpolymerized flagellin molecules into the culture supernatant. These mutants produced nonfunctional, severely truncated filaments that were not attached to the cell body.

Though the *V. vulnificus* flagellar gene locus shared same ORF arrangements and highest homologies with *V. parahaemolyticus*, phenotypes of the HAP1 and HAP3 mutants were somewhat different between the two species. Without either HAP1 or HAP3, in *V. vulnificus*, flagellation could not be observed at all under electron microscope. Any evidence of truncated flagellation could not be found in the entire electron microscope fields. Western blot analysis of the major flagellin FlaB in the cell pellets and culture supernatants also confirmed that the flagellins are not assembled properly at the hook in the HAP1 and HAP3 mutants. While FlaB in the cell pellets decreased, more FlaB appered to be secreted in the culture supernatants by the HAP1 or HAP3 mutation. HAP1 and HAP3 were equally important for the flagellation and motility.

To rule out polar effects of the HAP mutations, we constructed and tested in-frame mutant of HAP1 or HAP3 gene. The two mutants showed nearly identical phenotypes. This was the reason why we used the Vv-FlgL mutant for the LD_{50} test. Adhesion to epithelial cells is a prerequisite to the colonization and invasion of enteropathogenic bacteria. Generally, adhesion involves molecular interactions between specific adhesions and their receptors. Motility of the cells probably assists the bacteria to penetrate the mucous layer and gain access to the underlying epithelial cells. Adhesion of the mutants to HeLa cells in a cell culture model also decreased. And the mutants were also less cytotoxic to HeLa cells. There can be two explanations for the decrease

in the adhesion and cytotoxicity of the HAP protein mutants. One is that the polar flagellum itself could act as an adhesion apparatus of *V. vulnificus*. There is a report showing evidence that flagella of EPEC are directly involved in the adhesion of the bacteria to host epithelial cells (3). The other is that active motility propelled by the polar flagellum seems to be a prerequisite to action of professional adhesion molecules. The professional adhesion molecules, yet to be identified, might act only when the bacterial cells come to a close contact with host cells by the action of the polar flagellum. These are also probable that the two possibilities are all involved in the adhesion and cytotoxicity of *V. vulnificus*. Decreased adhesion and cytotoxicity resulting from the defect of flagellation in the mutants caused increase in LD_{50} to mice. These results suggest that the HAP proteins play an important role in polar flagellation and motility and polar flagellar propeller plays an important role in the pathogenesis of *V. vulnificus* infection.

In conclusion, the HAP proteins might play an important role in polar flagellation and motility mediated by the polar flagellum that is closely related to the virulence of *V. vulnificus*.

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