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Role of Flagellum and Motility in Pathogenesis of *Vibrio vulnificus*

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To assess the role of the flagellum which was detected by immunoscreening of surface proteins of *Vibrio vulnificus*, an *flgE*-deleted mutant was constructed and tested for its pathogenicity. The ability of this nonmotile mutant to adhere to INT-407 cells and its role in biofilm were decreased, as was its lethality to mice.

Vibrio vulnificus is a gram-negative bacterium that causes gastroenteritis and primary septicemia, especially in immunocompromised humans (11). Several virulence factors have been discovered in *V. vulnificus*, including the expression of lipopolysaccharide (1), capsular polysaccharide (21), elastase (6), and a phospholipase A_2 (20), as well as iron availability (22). Motility could be proposed to be another virulence determinant in addition to the aforementioned factors (3). *V. vulnificus* is a highly motile organism by virtue of a polar flagellum, as are the closely related vibrios. In *V. cholerae*, nonmotile mutants have been shown to accumulate less fluid in rabbit ligated ileal loops (17). Recently, a *V. vulnificus* mutant showing a decreased cytotoxicity to HeLa cells was found to have a transposon insertion at the *flgC* gene encoding a flagellar basal body (8).

We performed an experiment to identify bacterial surface molecules, which are required for the initiation of pathogenic interactions of *V. vulnificus* with a host. From an extensive screening process, a clone containing the *flgDEF* operon which encodes the components of the flagellum was obtained. Having constructed a knockout mutant of the *flgE* gene, we made a flagellum-deficient *V. vulnificus* mutant, and we then went on to investigate the role of flagellum-derived motility in the virulence of this pathogen to host cells.

Isolation of the *flgDEF* clone from immunoscreening of surface proteins of *V. vulnificus*. The strains and plasmids used in this study are listed in Table 1. To prepare whole-cell lysate, exponential-phase *V. vulnificus* ATCC 29307 was resuspended in 10 mM Tris-HCl (pH 7.4) and disrupted with an ultrasonic liquid processor (model XL2020 sonicator; Misonix). After ultracentrifugation (100,000 × *g*) for 1 h at 4°C, the pellet was added to 0.1% sodium lauryl sarkosinate in 7 mM EDTA. A sarkosyl-insoluble fraction (140 μg) was used for three consecutive immunizations of a rabbit. Ten days after the last injection, the blood of the immunized rabbit was collected and used for immunoscreening of the λZAPII-based expression library of *V. vulnificus*.

Approximately 20,000 plaques from the expression library were screened for clones interacting with the anti-surface protein serum described above. Five of the 11 candidate plaques showed reproducible immune reactions with the serum during further purification steps. The plasmids derived from the excision of these five clones, pBKH1 to pBKH5, were found to contain the identical DNA fragment; therefore, pBKH1 was used for further studies.

Restriction analysis of pBKH1 by using BamHI showed that it had an insert of 2.7 kb (Fig. 1A). The DNA insert of pBKH1 was found to contain a partial sequence of *flgD*, a complete sequence of *flgE*, and a partial sequence *flgF*, which encode the C-terminal region of a hook cap (12), an intact monomeric subunit of the hook (10), and the N-terminal region of the proximal rod (5), respectively.

Construction and characterization of *flgE* knockout mutant of *V. vulnificus*. Since the hook protein of the flagellum is located on the cell surface as a multimer, we investigated the function of *flgE*, one of three genes present in pBKH1, by constructing a *flgE* knockout *V. vulnificus* mutant. pBKH1 was digested with BglII and EcoRV, which reside within the *flgE* gene (Fig. 1A). This deletion resulted in the loss of an internal region of the FlgE protein from the 36th to the 324th amino acid residue. Next, the larger fragment was ligated to produce pBKΔ*flgE*. A DNA fragment of pBKΔ*flgE*, which included the *flgD′* Δ*flgE flgF′* region, was inserted into the corresponding sites of a suicide vector, pKAS32 (18). The resultant plasmid pKASΔ*flgE* in *Escherichia coli* strain SM10λ*pir* was mobilized to *V. vulnificus* ATCC 29307. Primary screening of the Δ*flgE* mutant was performed by PCR with two *flgE*-specific primers, FlgE-F (5′-CCCCTCGAGATGTCATATGTATCTTTAAGC G-3′) and FlgE-R (5′-GAATCTGCAGGATGTTCTG-3′). PCR of the mutant *V. vulnificus* resulted in the production of a 372-bp DNA fragment, while a 1,302-bp DNA fragment was produced from PCR of the wild-type *flgE* locus (Fig. 1B). The resultant *flgE* knockout mutant was named F34 and was used for further studies.

The aforementioned oligonucleotides FlgE-F and FlgE-R were used to amplify a 1.3-kb DNA fragment containing a full sequence of the *flgE* gene from the genomic DNA of *V. vulnificus*, and the resultant DNA was cloned to an expression plasmid, pGEX4T-1 (Phamacia). Recombinant FlgE protein

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10λ*pir*	*thi thr leu tonA lacy supE recA*::RP4-2-Tc::Muλ*pir*; OriT of RP4 Km^r; conjugational donor	9
XL1-blue MRF′	Strain used for genomic library construction	Stratagene
SLORL	Strain used for excision of library	Stratagene
BL21(DE3)	Strain B F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ(DE3)	Laboratory collection
V. vulnificus		
ATCC 29307	Clinical isolate	Laboratory collection
F34	ATCC 29307, Δ*flgE*	This study
Plasmids		
λZAPII	Vector used for construction of *V. vulnificus* genomic library	Stratagene
pBK-CMV	Phagemid derived from λZAPII	Stratagene
pBKH1	pBK-CMV, 2.7-kb *flgDEF* fragment of *V. vulnificus*	This study
pBKΔ*flgE*	pBK-CMV, 1.7-kb *flgD*Δ*EF* fragment of *V. vulnificus*	This study
pKAS32	Suicide vector; *oriR6K oriT* Ap^r *rpsL*	18
pKASΔ*flgE*	pKAS32, 1.7-kb *flgD*Δ*EF* fragment of *V. vulnificus*	This study
pGEX4T-1	Expression vector	Phamacia
pGEX*flgE*	pGEX4T-1, $flgE^+$	This study
pRK415	Broad-host-range plasmid	7
pRK::*flgF′ED′*	pRK415, 2.7-kb *flgD′EF′* fragment of *V. vulnificus*	This study

[a] GST, glutathione *S*-transferase.

was overexpressed in *E. coli* BL21(DE3) by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) at 1 mM and purified by using a glutathione affinity column as directed by the manufacturer (Phamacia). Purified FlgE protein (200 μg) was used to prepare FlgE-specific polyclonal antibodies by three consecutive immunizations into Sprague-Dawley rats at 3-week intervals. We then performed a Western blot analysis of the wild-type and F34 strains by using polyclonal antibodies against the recombinant FlgE. While the protein of ca. 50 kDa was found in the extract of the wild type, this protein was not detected in the extract of F34, suggesting that a disruption of the *flgE* gene abolished the expression of the putative FlgE protein in this strain (Fig. 2).

The *flgE* knockout mutant was then examined for the presence of a flagellum. Bacterial cells were negatively stained with 2% (wt/vol) phosphotungstic acid (pH 7.4) on a Formvar carbon-coated grid and observed with a transmission electron microscope (CM-10; Philips) operated at 75 kV. Observation with the transmission electron microscope clearly showed that wild-type *V. vulnificus* retained a polar flagellum (Fig. 3A) but that the mutant *V. vulnificus* had completely lost its flagellum (Fig. 3B).

To observe the swimming motilities of *V. vulnificus*, the wild-type and F34 strains were freshly grown in Luria-Bertani broth with 2.5% (wt/vol) NaCl (LBS) at 30°C with aeration to an optical density at 600 nm (OD_{600}) of 0.7, washed, and then resuspended in phosphate-buffered saline (PBS) to a final concentration of 10^6 CFU/ml. Spotting these cell suspensions (3 μl) on LBS medium containing 0.3% agar showed that the *flgE* strain did not demonstrate any motility and appeared as a small and sharply delineated cell mass, whereas wild-type *V. vulnificus* showed a distinct motile phenotype with a large diffuse spreading halo (data not shown). The degree of motility for each strain was presented in a quantitative model by measuring the diameters of spreading halos on a 0.3% LBS agar plate. The diameters of the bacterial halos for ATCC 29307 and F34 were 32.4 ± 1.7 and 5.4 ± 0.5 mm, respectively.

Functional analysis of the *flgE* gene in *V. vulnificus*. We examined the role of FlgE in the pathogenesis of *V. vulnificus* by using a mouse model. Specific pathogen-free, 7-week-old, female ICR mice were used in all experiments. Overnight cultures of various strains grown in LBN broth (Luria-Bertani medium with 0.86% [wt/vol] NaCl) were freshly cultivated in the same medium up to an OD_{600} of 0.7, harvested, washed once in PBS, and then resuspended in PBS–0.01% gelatin. One hundred microliters of serial dilutions of the bacterial suspension (containing from 1.4×10^2 to 1.4×10^7 *V. vulnificus* cells) was then injected intraperitoneally into six mice per dilution group. The numbers of dead mice were determined 48 h after the injection, and the 50% lethal dose (LD_{50}) was calculated with an equation provided by Reed and Muench (16). Mice infected with the wild type showed an LD_{50} of 4.4×10^4 cells, whereas mice injected with mutant *V. vulnificus* had a 10-fold higher LD_{50}, i.e., 5.0×10^5 cells.

The virulence of the *V. vulnificus* strains to mice was also measured under conditions such as excess iron, as described by Starks et al. (19). Prior to bacterial inoculation, iron dextran was injected into mice intraperitoneally at 250 μg/g of mouse. One hundred microliters of serial dilutions of the bacterial suspensions (containing from 10^2 to 10^8 *V. vulnificus* cells) was injected subcutaneously into six mice per dilution group. In case of infection with the wild type, the LD_{50} value was 1.0×10^3 bacteria. To obtain a rate of 50% for the deaths of mice injected by the mutant, 5,000 times more cells, 5.0×10^6 bacteria, were required.

Since the flagellum is known to be involved in motility as

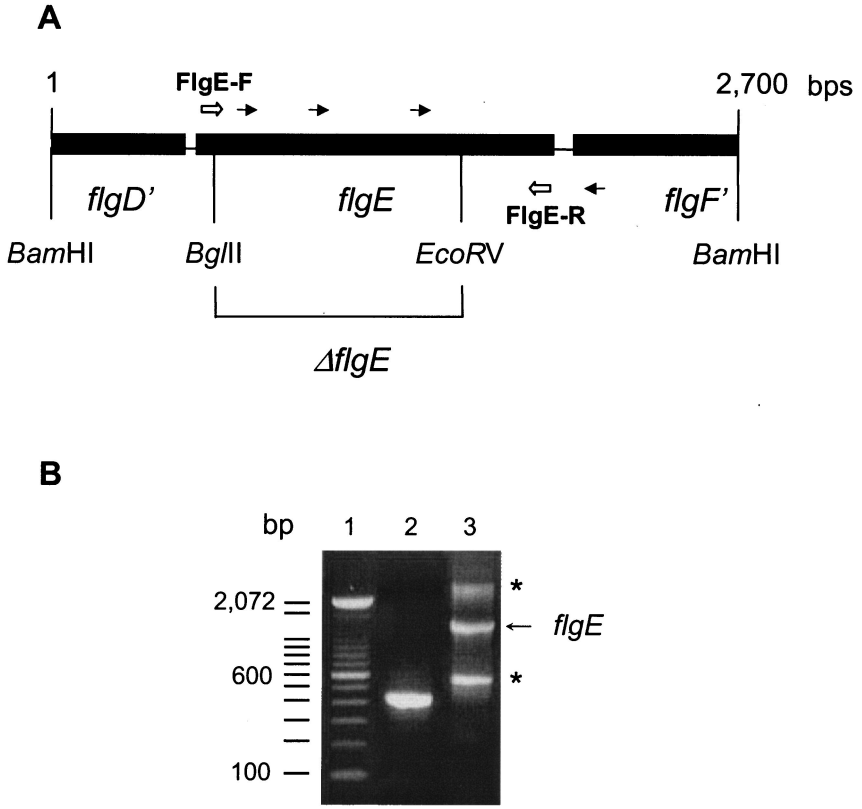

FIG. 1. Genetic organization of *V. vulnificus flgDEF* region in pBKH1 and confirmation of the *V. vulnificus flgE* knockout mutant F34. (A) The deleted region in pBKΔ*flgE* is indicated by restriction sites BglII and EcoRV. Primers for PCR used to examine the *flgE* loci, FlgE-F and FlgE-R, are indicated by open arrows. Black arrows represent locations of the internal primers used for sequence determination of this *flgDEF* region; (B) PCR was used to examine the *flgE* loci of the wild type and F34 with FlgE-F and FlgE-R primers. Lane 1, DNA size marker (100-bp ladder); lane 2, PCR product of F34; lane 3, PCR product of wild-type *V. vulnificus*. The wild-type *flgE* DNA band is indicated with an arrow, and the DNA bands labeled with asterisks are the nonspecific PCR products.

well as in attachment to host cells by other pathogenic bacteria (14), we examined the role of flagellum in the adherence of *V. vulnificus* to the cell line. Adherence assays were performed with INT-407 cells (ATCC CCL-6) derived from human intestinal epithelium. Each well on 24-well culture plates was seeded with about 10^5 INT-407 cells and grown overnight at 37°C in the presence of 5% CO_2. The cell lines for the assay were prepared by removing the medium, washing them twice with Hank's balanced salt solution, and then adding 1 ml of serum-free minimal essential medium with Earle's salt. Cell monolayers were then inoculated in triplicate with 50 μl of the diluted bacterial cells grown overnight in LBS broth to give a multiplicity of infection of ca. 5 and were incubated at 37°C in 5% CO_2 for 15 min. The monolayer was then washed six times with prewarmed PBS to remove nonadherent bacteria. Following the last wash, the INT-407 cells were broken with 0.1% Triton X-100 solution for 15 min. The bacteria were recovered and plated on LBS agar plates. The number of input bacteria was also determined by plating diluted bacterial cultures onto LBS agar plates. The limit of detection of adhered bacteria with the present method was 10^3 *V. vulnificus* cells, since inoculations of bacterial cells less than 10^3 did not recruit any bacterium as adherent cells. In adherence assays, the *flgE* mutant showed a severe defect in adherence to the cells. While 5.4% of the added wild-type bacteria was found to be adherent to INT-407 cells, only 0.12% of the initially added F34 bacteria was recovered when adhered to INT-407 cells (Fig. 4A).

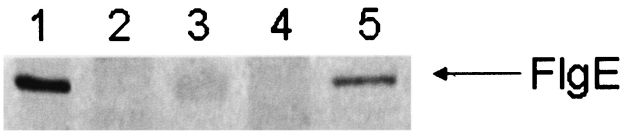

FIG. 2. Western blot analysis with polyclonal antibodies against the recombinant FlgE protein. Lane 1, crude extract of wild-type *V. vulnificus* ATCC 29307; lane 2, crude extract of F34; lane 3, protein size markers; lane 4, crude extract of F34 harboring pRK415; lane 5, crude extract of F34 harboring pRK::*flgF'ED'*. The arrow indicates the FlgE protein of 47.7 kDa.

We also examined the role of FlgE in biofilm formation. This assay was based on the ability of bacteria to initiate biofilm formation on a polystyrene surface and was performed as previously described (13). Overnight cultures were inoculated at a dilution of 1:20 in fresh AB medium (0.3 M NaCl, 0.05 M $MgSO_4 \cdot 7H_2O$, 0.2% vitamin-free Casamino Acids, 0.01 M potassium phosphate [pH 7.0], 1 mM L-arginine, 1% glycerol)

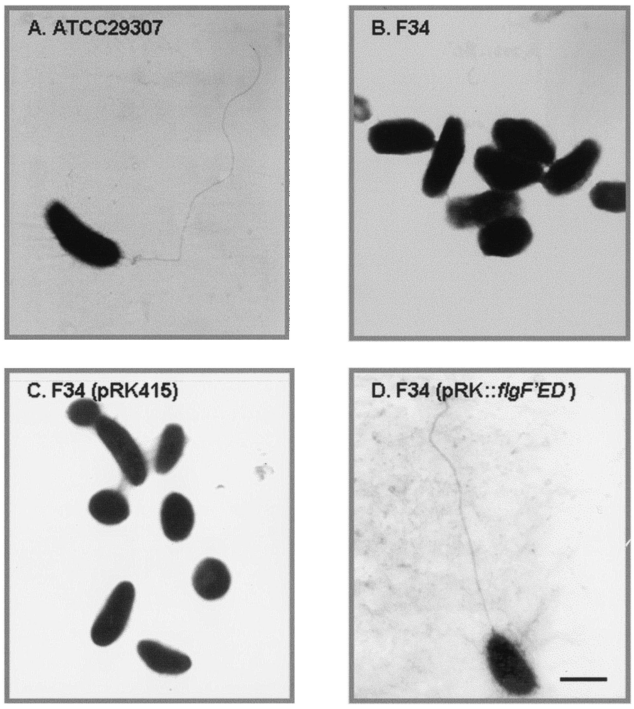

FIG. 3. Transmission electron micrographs of *V. vulnificus* strains negatively stained with potassium phosphotungstic acid. (A) *V. vulnificus* ATCC 29307; (B) *V. vulnificus* ATCC 29307 isogenic *flgE* mutant F34; (C) F34 harboring pRK415; (D) F34 harboring pRK::*flgF'ED'*. The bar (D) represents a length equivalent to 1 μm.

(4), which was found to be a favorable condition for biofilm formation by *V. vulnificus* (C.-B. Kim, S.-J. Park, and K.-H. Lee, unpublished data). At various times, the cultures were monitored for planktonic growth by measuring the OD_{595}, and suspended bacterial cells were removed. The wells were subsequently washed with artificial seawater (0.1 M $MgSO_4 \cdot 7H_2O$, 0.02 M $CaCl_2 \cdot 2H_2O$, 0.4 M NaCl, 0.02 M KCl, 0.05 M Tris-HCl [pH 8.0]) (2) three times, and the bacterial cells on the surface were stained with 1.0% crystal violet (CV) for 30 min, washed, and dried. Biofilm formation was quantified by solubilizing stains with 100% ethanol and measuring absorbance using a plate reader at 550 nm (series 700 microplate reader; Cambridge Technology). Readings were corrected by subtracting the values obtained from wells containing AB medium only, and the results are presented as a ratio of the OD_{550} to OD_{595}. As shown in Fig. 5A and B, wild-type *V. vulnificus* formed more CV-staining mass than did F34. We also observed biofilm formations of F34 and the wild type in a rather direct way. Both wild-type *V. vulnificus* and F34 were also grown in AB medium for 72 h in the presence of glass wools (8-μm diameter; Sigma), stained with 0.1% CV, and then observed for biofilm formation with a light microscope. In good agreement with biofilm formation determined by the former method, wild-type *V. vulnificus* formed a bacterial mass on the surface of the glass wool, whereas a much smaller number of F34 cells was observed under the same conditions (Fig. 5C and D).

Complementation of the *flgE* gene to F34. The *flgE* mutant *V. vulnificus* was provided with the intact *flgE* gene in a broad-host-range vector. A 2.7-kb BamHI fragment containing the intact *flgE* gene was isolated from pBKH1 and was cloned into pRK415 (7) to produce pRK::*flgF'ED'*. Strain F34 containing pRK415 was also prepared as a control strain. Expression of FlgE protein in the complemented strain was shown in a Western blot by using FlgE polyclonal antibodies, whereas the FlgE protein was not detected in the control strain (Fig. 2). During this experiment, we found that pRK::*flgF'ED'* was easily cured of *V. vulnificus* if tetracycline was not provided in the media. Therefore, the strains were maintained in the presence of tetracycline (3 μg/ml) in the following experiments.

Negative staining of bacterial cells demonstrated the formation of a flagellum in a complemented strain, whereas the

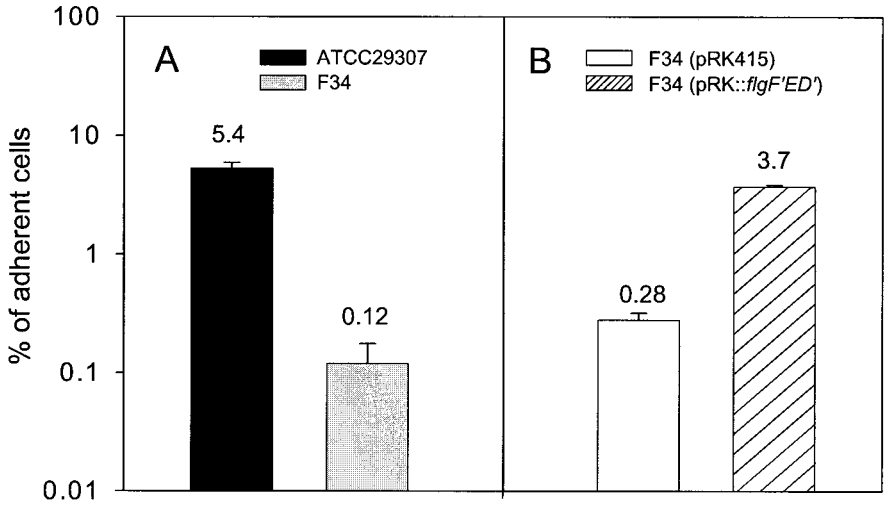

FIG. 4. Adherence of *V. vulnificus* to the INT-407 cell line. (A) Adherence of wild-type *V. vulnificus* ATCC 29307 and the *flgE* knockout mutant F34. (B) Adherence of F34 harboring pRK415 and F34 harboring pRK::*flgF'ED'*. Adherence values are indicated as the percentage of the numbers of bacterial cells that adhered to INT-407 to those of bacteria initially added to INT-407. Each experiment was repeated at least three times, and the average values are indicated with bars showing standard deviations.

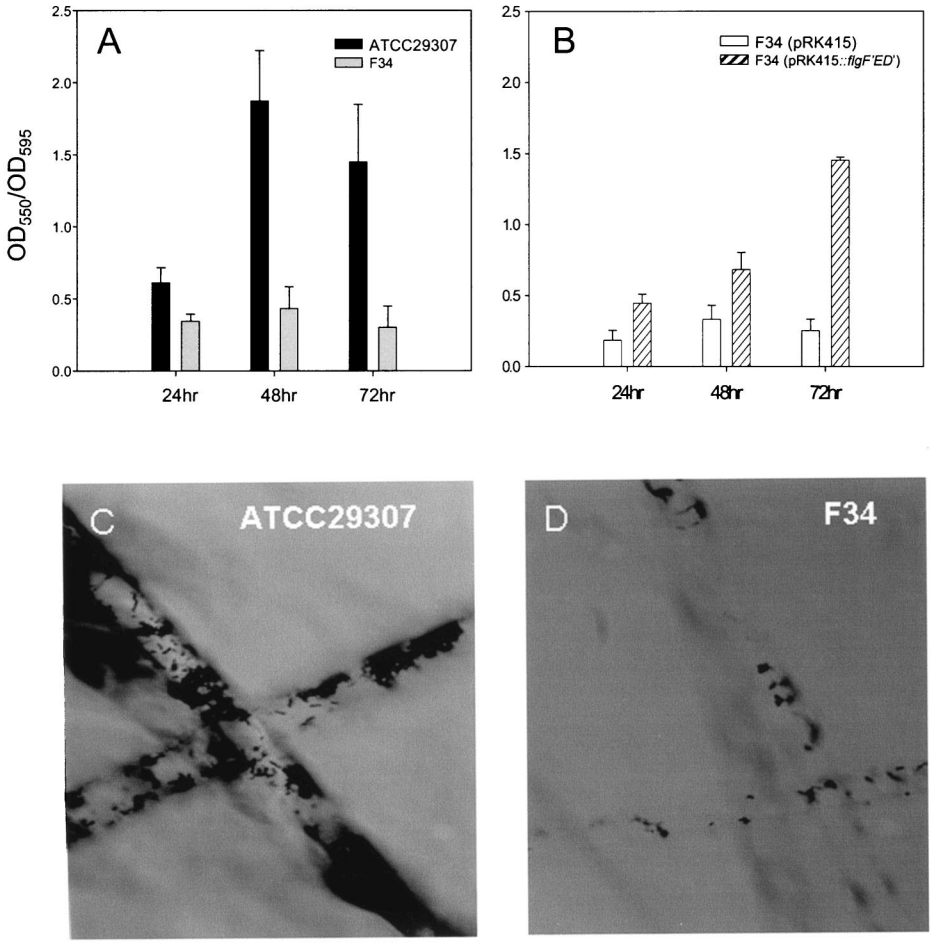

FIG. 5. Biofilm formation of *V. vulnificus.* The determination of biofilm formation of wild-type *V. vulnificus* ATCC 29307 and the *flgE* knockout mutant F34 (A) and biofilm formation of F34 harboring pRK415 and F34 harboring pRK::*flgF′ED′* (B) on a polystyrene surface by the CV staining method is shown. Biofilm formation was quantified by measuring absorbance using a plate reader at 550 nm (OD_{550}) and are presented as values normalized by planktonic cell density (OD_{595}). Each experiment was repeated at least six times, and the average values were indicated with bars showing standard deviations. Light microscopic observation of biofilm formation of wild-type *V. vulnificus* ATCC 29307 (C) and of *flgE* knockout mutant F34 (D) on glass wool is shown.

control strain was unable to make a flagellum (Fig. 3C and D). The bacterial motilities of these two strains were examined by incubating them on 0.3% LBS agar supplemented with tetracycline. Motility was restored when the intact *flgE* gene was added to the *flgE* mutant pRK::*flgF′ED′*. For ATCC 29307 and F34 alone, bacterial cells were grown in LBS containing tetracycline (3 μg/ml) at 30°C for 16 h, because their growth was retarded in the presence of tetracycline. The diameters of the bacterial halos for F34 with pRK415 and F34 with pRK:flgF′ED′ were 5.3 ± 0.5 and 37.5 ± 1.9 mm, respectively.

In respect to bacterial adherence to INT-407 cells, the ability of complemented F34 was increased compared to that of the control strain F34 harboring pRK415 (Fig. 4B). The ability to form a biofilm was also examined (Fig. 5B). F34 with pRK415 was not able to develop biofilms on a polystyrene surface. On the other hand, the ability to form biofilm was restored in F34 with pRK::*flgF′ED′*. However, mortality of mice did not show a distinct effect of *flgE* complementation, showing an LD_{50} of the complemented F34 similar to that of the control strain (5.6×10^5 versus 7.5×10^5). A failure to complement the mutant phenotype in F34 with pRK::*flgF′ED′* might stem from a loss of the plasmid in mice where it is free of antibiotic selection pressure. It has been reported by Paranjpye et al. (15) that partial complementation of mortality by a *V. vulnificus* mutant was due to a loss of plasmid containing a corresponding gene.

We found that this flagellum-deficient *flgE* mutant of *V. vulnificus* showed a significant decrease in its virulence to mice compared to that of the wild type. The flagellum of *V. vulnificus* seems to perform a function in cytoadherence, since F34 was significantly impaired in its ability to adhere to the cell line used (Fig. 4A). This result implies that the motility of *V. vulnificus* may be required for localization to the sites of infection and that the flagellar apparatus may serve as an adhesin or as a structure for invasion into the host cells. The exact identification and characterization of adhesin molecules involved in *V. vulnificus*-host cell interactions will be a primary aspect of our future investigation.

Nucleotide sequence accession number. The nucleotide sequence of the isolated clone has been deposited in GenBank under the accession number AY147417.

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