Characterization of Two Outer Membrane Proteins, FlgO and FlgP, That Influence *Vibrio cholerae* Motility[▽]

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Vibrio cholerae is highly motile by the action of a single polar flagellum. The loss of motility reduces the infectivity of *V. cholerae*, demonstrating that motility is an important virulence factor. FlrC is the sigma-54-dependent positive regulator of flagellar genes. Recently, the genes VC2206 (*flgP*) and VC2207 (*flgO*) were identified as being regulated by FlrC via a microarray analysis of an *flrC* mutant (D. C. Morris, F. Peng, J. R. Barker, and K. E. Klose, J. Bacteriol. 190:231–239, 2008). FlgP is reported to be an outer membrane lipoprotein required for motility that functions as a colonization factor. The study reported here focuses on the characterization of *flgO*, the first gene in the *flgOP* operon. We show that FlgO and FlgP are important for motility, as strains with mutations in the *flgOP* genes have reduced motility phenotypes. The *flgO* and *flgP* mutant populations display fewer motile cells as well as reduced numbers of flagellated cells. The flagella produced by the *flgO* and *flgP* mutant strains are shorter in length than the wild-type flagella, which can be restored by inhibiting rotation of the flagellum. FlgO is an outer membrane protein that localizes throughout the membrane and not at the flagellar pole. Although FlgO and FlgP do not specifically localize to the flagellum, they are required for flagellar stability. Due to the nature of these motility defects, we established that the flagellum is not sufficient for adherence; rather, motility is the essential factor required for attachment and thus colonization by *V. cholerae* O1 of the classical biotype. This study reveals a novel mechanism for which the outer membrane proteins FlgO and FlgP function in motility to mediate flagellar stability and influence attachment and colonization.

The causative agent of the infectious diarrheal disease cholera is the bacterium *Vibrio cholerae*. This enteric pathogen naturally inhabits an aquatic environment and is motile by the action of a single polar flagellum. The mechanism of pathogenesis by which *V. cholerae* causes disease is a stepwise and cyclic process. First, contaminated food or water is ingested; then, the bacteria pass through the stomach. After withstanding that acidic environment, the bacteria enter the lumen of the intestine and attach to the intestinal epithelium of the human host. Once the bacteria establish an initial attachment, the bacteria begin to express the toxin-coregulated pilus, which facilitates bacterium-bacterium interactions and the formation of microcolonies. Subsequently, an enterotoxin, cholera toxin, is expressed and secreted by the bacteria, which through a cascade of events leads to profuse, watery diarrhea. Eventually, the bacteria are shed from the host and are dispersed back into the environment, thus renewing the cycle.

Although the later stages of the disease, which include the production of toxin-coregulated pilus and cholera toxin, are well characterized, little is known about the initial stages of pathogenesis. The attachment factor GbpA has been shown to bind chitin, an abundant substance found in the environment that is a polymer of *N*-acetylglucosamine (GlcNAc) carbohydrate subunits (17). GbpA is also involved in the initial attachment of *V. cholerae* to epithelial cells, which have exposed

GlcNAc moieties on their surfaces. In the absence of *gbpA*, only a 50% reduction in attachment is observed, suggesting that there may be additional factors that are involved in initiating attachment to the intestinal epithelium.

Colonization requires a functional flagellum; therefore, motility is a crucial virulence factor of *V. cholerae* (9, 12). The flagellum functions to propel the cell through its environment. In the host, the flagellum functions as a means to gain access to the microvilli of the epithelial cells by facilitating the penetration of the intestinal mucus layer, an action that is important for colonization (1, 4, 14). The flagellum is composed of a base, a hook, and a filament. The flagellar apparatus is assembled in an ordered cascade of events. The genes necessary for motility and chemotaxis are found distributed among six regions on the large chromosome of *V. cholerae* (regions I to VI) (28). The expression of these genes is under the control of a tightly regulated transcriptional hierarchy. The assembled flagellum is powered by sodium motive force, and rotation of the flagellum leads to swim speeds of up to 65 $\mu\text{m/s}$ (20, 30). Additionally, chemotaxis controls the bidirectional rotation of the flagellum, which also influences the virulence of *V. cholerae* (4).

The flagellum is encased in a protective sheath, which appears to be contiguous with the outer membrane (OM) and contains lipopolysaccharide and protein (7, 13, 31). Although lipopolysaccharide is associated with the sheath, it has not been concluded whether the sheath is indeed a continuation of the OM or a separate entity. The flagellar sheath of *V. cholerae* has been widely studied; however, its origin and function are not completely understood. It has recently been proposed that the flagellar sheath of *V. cholerae* functions as a means to evade

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host immune responses (36). The sheath was also shown to protect the filament from dissociation when exposed to high temperatures and low pH, conditions that easily dissociate the nonsheathed flagellum of *Salmonella enterica* serovar Typhimurium (36). Furthermore, the flagellum is also thought to have adhesive properties, which would allow it to function as an attachment factor (1, 9, 14, 29).

Pilus-mediated-attachment, motility, nitrogen metabolism, alginate production, glutamine synthesis, and type III secretion genes are regulated by RpoN (σ^{54}), an alternate sigma factor, in various pathogenic gram-negative bacteria (16). In *V. cholerae*, RpoN plays an important role in positively regulating flagellar gene expression. A *V. cholerae* *rpoN* mutant is aflagellated and displays a 10- to 20-fold reduction in its ability to colonize the infant mouse intestine compared to the wild-type (WT) strain (19). However, the nonflagellated *flaA* mutant shows only a twofold reduction in the same infant mouse competitive assay, suggesting that RpoN regulates the expression of additional factors other than flagella that are important for attachment and colonization. Given that RpoN appears to play a major role in the ability of *V. cholerae* to adhere to the intestinal epithelium, we chose to investigate other genes regulated by RpoN that might contribute to intestinal colonization.

A recent study of *flrC*, which encodes the RpoN-dependent transcriptional activator of the class III flagellar genes, identified the genes VC2207 (*flgO*) and VC2206 (*flgP*) via a microarray analysis of an *flrC* mutant of the classical biotype strain O395 (25). The authors report the effects of Δ *flgP* on motility and colonization. The deletion of *flgP* resulted in the production of abnormal flagella that were jagged and lacked the characteristic sinusoidal curvature of the flagellar structure, resulting in the loss of motility. Intriguingly, FlgP is an OM lipoprotein, in which lipidation at residue C18 is essential for localization to the OM, as well as improved colonization, but not motility. A new and contradicting report by Cameron et al. states that an *flgP* mutant of the El Tor biotype strain C6706 displays normal flagella (5).

Whereas FlgP was the primary focus of the study by Morris et al. (25), the work reported here encompasses an initial characterization of FlgO as well as additional findings with respect to FlgP in the classical biotype strain O395. We have determined that both FlgO and FlgP play a role in motility and intestinal colonization. The *flgO* and *flgP* mutant strains exhibit lower numbers of motile cells and have fewer flagella. We also determined that the flagella produced by the *flgO* and *flgP* mutant strains were normal in appearance except for their length. Transmission electron microscopy studies revealed truncated flagella that averaged half the length of the WT flagella. Interestingly, inhibition of the flagellar motor restored the length of the flagellum to the length of the WT flagella, suggesting a stability defect, which is further supported by increased sensitivity of the flagella elaborated by the *flgO* and *flgP* mutant strains to high temperature and low pH. Additionally, we demonstrated that FlgO localizes to the OM and that neither FlgO nor FlgP requires the other for proper localization. Further localization studies indicate that FlgO and FlgP are present throughout the membrane and not only at the flagellar pole. Also, unlike other known OM proteins (OMPs), FlgO and FlgP associate primarily with the cell and not the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>E. coli</i> strain S17-1 λ pir	Tp ^r Sm ^r <i>recA thi pro</i> <i>hsdR</i> ⁺ [RP4-2Tc::Mu::Km ^r Tn7] (λ pir)	Laboratory collection 27
<i>V. cholerae</i> strains		
O395	Classical Ogawa, Sm ^r	Laboratory collection
KSK258	O395 <i>lacZ</i>	Karen Skorupski
MD21	O395 Δ <i>flgO</i>	This study
MD22	O395 Δ <i>flgP</i>	This study
MD23	O395 Δ <i>flgOP</i>	This study
RM369	O395(pKAS178)	This study
RM374	MD21(pRMM17)	This study
RM376	MD23(pRMM17)	This study
RM400	MD22(pRMM18)	This study
RM401	MD23(pRMM18)	This study
RM407	MD21(pRMM17, pBAD22)	This study
RM408	MD22(pRMM18, pBAD22)	This study
TJK217	O395 Δ <i>rpoN</i>	This study
Plasmids		
pBAD22	Amp ^r	10
pKAS32	Amp ^r	32
pKAS178	Kan ^r	21
pMD18	pKAS32 Δ <i>flgO</i>	This study
pMD19	pKAS32 Δ <i>flgP</i>	This study
pMD20	pKAS32 Δ <i>flgOP</i>	This study
pRMM17	pKAS178 FlgO-His ₆	This study
pRMM18	pKAS178 FlgP-His ₆	This study
pTK30	pKAS32 Δ <i>rpoN</i>	This study

flagellar fraction. The results presented here provide a novel mechanism by which the OMPs FlgO and FlgP function in motility to influence the stability of the flagellum of *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and DNA manipulations. The strains and plasmids used in this study are listed in Table 1. All cultures were grown in LB medium supplemented with antibiotics and arabinose when needed at 37°C. The following antibiotics were used at the indicated concentrations: ampicillin, 100 μ g/ml; streptomycin, 100 μ g/ml; kanamycin, 45 μ g/ml. When required, arabinose was added to the medium at concentrations of 0.02 to 0.2%. All DNA manipulations were performed using standard molecular and genetic techniques (2, 23).

RNA isolation and microarray analyses. Two independent experiments were performed as follows: *V. cholerae* O395 and the Δ *rpoN* mutant were grown overnight at 37°C with aeration. The cultures were then diluted 1:100 into fresh medium and further incubated to an optical density at 600 nm (OD₆₀₀) of ~1.2. RNA isolation, cDNA probe labeling, microarray hybridization, and microarray data analyses were carried out as previously described (21).

Motility assays and phase-contrast microscopy. Motility plates (LB with 0.3% agar) were inoculated to test motility phenotypes. Colonies grown on solid media were stabbed to the bottom of the soft agar plate using sterile, round toothpicks. Plates were incubated at 37°C for 8 to 12 h. By measuring the area of motility using the ImageJ software (<http://rsb.info.nih.gov/ij/>), we calculated the area ratios (mutant to WT) for each strain, which allowed the quantification of the motility phenotypes. For visualizing motility via phase-contrast microscopy, overnight cultures were diluted 1:100 into fresh media and grown to mid-log phase (OD₆₀₀ of ~0.6). A 1:20 dilution was made on a glass slide for observation by phase-contrast microscopy. OpenLab software was used to obtain time-lapse images, which were converted to QuickTime movies. The number of motile cells was determined and reported as a percentage of the total population.

Transmission and immunoelectron microscopy. Negatively stained samples were prepared as previously described with modification (34). Overnight cultures

were diluted 1:100 into fresh media and grown to mid-log phase (OD_{600} of ~ 0.6). Formvar-coated grids were incubated with cultures for 2 min. The grids were then fixed in a 4% paraformaldehyde solution and washed three times for 1 min each in 0.1 M sodium cacodylate. Whole-cell preparations were negatively stained with 0.5% phosphotungstic (PTA) acid (pH 6.5) for 2 min. Thin sections were immunolabeled by blocking in 3% (wt/vol) casein in Tris-buffered saline (15). A 1:10 dilution of the primary antibody was incubated for 1 h and then washed with Tris-buffered saline containing Tween 20. The gold-conjugated secondary antibody was diluted 1:25 and incubated for 1 h in the dark.

Subcellular fractionation, SDS-PAGE, and immunoblot analysis. Whole-cell extracts were prepared by suspending overnight cultures in $2\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and were boiled for 10 min. $2\times$ SDS-PAGE without 2-mercaptoethanol and bromophenol blue was used for protein estimation. Subcellular fractionation was performed as previously described (35). Protein concentrations of whole-cell extracts and subcellular fractions were determined with a BCA protein assay kit (Pierce). Samples containing equal amounts of total protein were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes at 4°C. All primary antibodies (anti-flagellin, anti-His, anti-OmpA, anti-OmpS, anti-OmpU, anti-OmpW, and anti-ToxR) were used at a dilution of 1:1,000. The horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse antibody) were used at a dilution of 1:10,000. Enhanced chemiluminescence detection reagents were utilized to visualize reactive bands (Amersham Pharmacia).

Tissue culture and bacterial attachment assay. HT-29 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and $1\times$ Pen-Strep (Gibco) under 5% CO_2 , as previously described (3, 18). Overnight bacterial cultures were diluted 1:100 in phosphate-buffered saline (PBS) before incubation with confluent HT-29 monolayers. The bacterial suspension was added to the epithelial cells and centrifuged for 10 min at $1,000\times g$ to ensure bacterium-epithelial cell interaction. The bacterial cells were allowed to attach to the HT-29 cells for 1 h at 37°C. The HT-29 monolayers and attached bacteria were washed three times with PBS to remove unattached bacterial cells. The HT-29 cells were then lysed to enumerate the attached bacteria by incubating the coculture for 30 min in a 1% Triton X-100 solution. The resulting bacterial suspension was serially diluted and plated for enumeration of bacteria on LB medium plates supplemented with streptomycin and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (40 μ g/ml) when required for competitive attachment assays.

Infant mouse cholera model. As previously described, test strains were mixed with equal numbers (1:1) of a Δ lacZ O395 reference strain (18). Four- to 6-day-old CD-1 mice from mixed litters were orally inoculated with 50 μ l of a 1×10^{-2} dilution of the bacterial mixture and incubated at 30°C for 24 h. The bacteria were then recovered by homogenizing harvested intestines with a Tissue Tearor in 4 ml of LB broth containing 10% glycerol. The homogenate was serially diluted and plated on LB agar plates containing streptomycin and X-Gal. The competitive index was calculated using the equation [test (output/input)]/[reference (output/input)]. Animal experiments were done in compliance with institutional animal care and use guidelines.

Isolation of crude flagellar fractions and flagellar treatments. Broth cultures were diluted 1:100 in fresh medium and grown overnight. The cells were pelleted by centrifugation at $10,000\times g$, and the supernatant was discarded. The cells were then suspended in PBS, vortexed for 2 min to shear the flagella from the cells, and centrifuged. The supernatant was removed and subjected to ultracentrifugation at $208,000\times g$ for 60 min to pellet the flagella. The resulting crude flagellar pellet was suspended in PBS. Flagella were kept at room temperature (no treatment) or treated at 50°C or 80°C for 10 min, followed by incubation at 4°C until needed. Equal volumes of flagella and buffered 50 mM sodium citrate solutions (pH 7, 5, and 3) were incubated at room temperature for a treatment time of 10 min as previously described (36). Following treatment, flagellar samples were loaded on a 4 to 20% polyacrylamide gel and electrophoresed under native conditions.

Microarray data accession number. The microarray data discussed herein have been deposited in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE17144.

RESULTS

RpoN regulates the expression of the genes VC2206 and VC2207. Because the *rpoN* mutant shows a greater colonization defect than a *flaA* mutant in an in vivo competitive index, we were interested in identifying the gene(s) regulated by

RpoN that might contribute to attachment and colonization of *V. cholerae* (19). RpoN is known to regulate the expression of genes involved in flagellar motility, glutamate synthesis, and a possibly unknown colonization factor(s) in *V. cholerae* (19). In an attempt to identify such novel factors, a Δ *rpoN* microarray analysis was carried out. In two separate microarray studies, a total of 139 genes showed changes in expression in the Δ *rpoN* mutant of at least threefold compared to that of the WT. Of the 139 genes, 86 genes were upregulated, whereas 53 genes were downregulated compared to the expression in the WT. We were interested in gene products that contained signal sequences, which is a common characteristic of attachment and colonization factors. The genes VC2206 and VC2207 appeared to be positively regulated by RpoN. The microarray data showed an average change of 18.79 ± 0.93 -fold in gene expression for VC2207 and an average change of 5.35 ± 1.32 -fold for VC2206. Independent of this study, Morris et al. recently reported that the genes VC2206 and VC2207 compose an operon positively regulated by FlrC, an RpoN-dependent flagellar regulatory protein (25). The genes VC2207 and VC2206 were named *flgO* and *flgP*. Our findings corroborate those reported by Morris et al. that RpoN regulates the gene expression of *flgOP* (25).

***flgO* and *flgP* are important for proper motility function in *V. cholerae*.** The *flgOP* operon can be considered to lie within an extension of the original flagellar gene cluster, a region consisting of flagellar structural genes, chemotaxis genes, and various hypothetical genes (25, 28). Given that FlgP plays an important role in motility in *V. cholerae*, as well as in *Campylobacter jejuni*, we next chose to investigate the role of FlgO in *V. cholerae* motility (25, 33). According to the data resulting from motility agar assays, the deletion of *flgO* resulted in approximately a 60% reduction in motility compared to that of the WT (Fig. 1A and B). The Δ *flgP* and Δ *flgOP* strains displayed even greater reductions in motility ($\sim 90\%$). Nonetheless, the *flgP* and *flgOP* mutants were significantly more motile than the *flaA* mutant. The motility defect of the *flgO* mutant can be partially complemented by plasmid-encoded FlgO-His₆ in soft agar (Fig. 1C). Likewise, expressing FlgP-His₆ in the *flgP* mutant increased the zone of motility (Fig. 1D). In addition, the *flgO* and *flgP* mutants appear to be epistatic, since the double mutant has the same phenotype as the Δ *flgP* mutant, suggesting that they operate in the same pathway. Thus, *flgOP* seem to be required for optimal motility of *V. cholerae*.

The *flgO* and *flgP* mutants exhibit low numbers of motile cells. Given that the Δ *flgP* motility agar phenotype suggested that motility is not completely lost, we observed the motility of the Δ *flgO* and Δ *flgP* broth cultures under phase-contrast light microscopy. Motile cells were enumerated, and the proportion of motile cells for each strain was quantified (Fig. 2A). Thirty-four percent of the WT cells were observed to be motile, whereas only 17% of the Δ *flgO* population was motile, which is a 50% reduction (17% versus 34%) in motility compared to the WT strain. An even greater reduction was discovered for the Δ *flgP* strain, where only 6% of the population was motile compared to 34% for the WT strain (an 82% reduction). The decreased percentage of motile cells in the *flgO* and *flgP* mutant populations observed under the light microscope is consistent with the motility agar data (Fig. 1).

FIG. 1. $\Delta flgO$ and $\Delta flgP$ mutants are defective in motility. (A) Area of motility of the WT (O395) and $\Delta flaA$ (nonmotile), $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ *V. cholerae* strains in motility agar. (B) Graphical representation of the area of motility shown in panel A. The NIH ImageJ software was used to measure the area of the zone of motility of each strain in quadruplicate. Brackets indicate statistical comparisons (*t* tests) between specific data sets; otherwise, asterisks indicate comparisons between the WT and the indicated strain. ***, $P < 0.0001$; **, $P < 0.005$. (C) The $\Delta flgO$ mutant can be partially complemented by a C-terminal His-tagged version of FlgO but not by the vector alone (0.02% arabinose). (D) Similarly, the $\Delta flgP$ mutant can be partially complemented by a C-terminal His-tagged version of FlgP but not by the vector alone (0.2% arabinose).

The *flgO* and *flgP* mutants produce fewer flagella than the WT. Because the *flgO* mutant and *flgP* mutant strains displayed a decline in the percentage of motile cells, we hypothesized that reduced rotation of the flagellum, a damaged filament, or perhaps the lack of a flagellum could explain why a population would show decreased numbers of motile cells compared to the WT. To test this, transmission electron microscopy was used to visualize the flagella of the *flgO*, *flgP*, and *flgOP* mutant strains. There were no visible abnormalities in the sheathing of the flagella; the flagella appeared to be smooth and not rough as previously reported (25) (Fig. 2B). One hundred cells were then analyzed for the presence of flagella. As expected, almost all of the WT cells had intact flagella (90%), whereas none of the nonmotile *flaA* mutant cells had flagella (Fig. 2C). More than half of the $\Delta flgO$ cells were flagellated (59%), with significantly fewer $\Delta flgP$ and $\Delta flgOP$ cells maintaining their flagella (41% and 42%, respectively). These results indicate that these mutant strains are still flagellated, but at a lower level than the WT. In the case of the *flgP* mutant, a large portion of these flagellated cells are not motile. This suggests a defect in either the rotation or the integrity of the flagellum.

The $\Delta flgO$ and $\Delta flgP$ mutants have truncated flagella. Although the *flgO* and *flgP* mutant flagella appeared smooth and sinusoidal like the WT flagella, we did observe variations in flagellar length. In order to study the potential differences in the flagellar structures produced by the *flgO* and *flgP* mutants, the ImageJ software was utilized to directly measure the length of flagella from electron micrographs. The average length of a WT flagellum was found to be 4.5 μm , with a range of lengths between 2.0 and 6.5 μm (Fig. 3). The average lengths of the *flgO*, *flgP*, and *flgOP* mutants were 2.5 μm . In other words, the majority of the flagella produced by the mutant strains were shorter than those of the WT. However, a small proportion of the mutant cells had flagella of lengths comparable to those of WT flagella ($\geq 4.5 \mu\text{m}$), indicating that these strains are capable of making full-length flagella. These data show that the *flgO* and *flgP* mutant strains are not able to properly modulate the length of the flagellum, which would imply a role for *flgO* and *flgP* in the stabilization of the flagellum.

Because we have observed full-length flagella among the *flgO* and *flgP* mutants, we hypothesize that the shorter flagella are the result of breakage. Rotation of the flagellum requires

FIG. 2. Motility defects correspond to a low percentage of motile cells. (A) The WT (O395) and mutant ($\Delta flgO$ and $\Delta flgP$) *V. cholerae* strains were observed for the presence or absence of motility under a light microscope, and 200 cells per strain were counted during several time-lapse movies. Brackets indicate statistical comparisons (*t* test) between specific data sets; otherwise, asterisks indicate comparisons between the WT and the indicated strain. ***, $P < 0.0001$. (B) Transmission electron micrographs showing that the flagella of the $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ strains are normal compared to that of the WT. All electron micrographs are at a magnification of $\times 4,000$. (C) The number of bacteria that maintained their flagella was determined under a transmission electron microscope. A total of 100 cells per strain (WT and $\Delta flaA$, $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ mutants) were examined. Brackets indicate statistical comparisons (*t* test) between specific data sets; otherwise, asterisks indicate comparisons between the WT and the indicated strain. ***, $P < 0.0001$; **, $P < 0.005$.

torque, a twisting force that has the potential to break an unstable filament. To test the hypothesis that the flagella assembled by the *flgO* and *flgP* mutants are more fragile than those of the WT strain, we introduced a $\Delta motX$ mutation into the $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ strains. MotX is an OMP that functions as part of the stator of the sodium-driven flagellar motor and is required for rotation of the flagellum in *V. cholerae* (8). Therefore, a *motX* mutant is nonmotile due to the lack of motor function. Additionally, the $\Delta motX$ strain appears to

FIG. 3. The $\Delta flgO$ and $\Delta flgP$ mutants produce short flagella. Transmission electron micrographs of the $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ strains in WT and *motX* backgrounds were utilized to measure the lengths of the flagella. A total of 25 cells per strain were analyzed using the ImageJ software. Asterisks indicate statistical comparisons (*t* test) between the WT and the indicated strain. ***, $P < 0.0001$; *, $P < 0.05$.

produce normal flagella in both appearance and numbers (percent flagellated), as MotX is not required for flagellar assembly (8). Also, the $\Delta motX$ mutation did not alter the flagellar length of the WT strain (Fig. 3). In contrast, the $\Delta motX$ mutation resulted in increased flagellar length produced by the $\Delta flgO$ and $\Delta flgP$ strains. The flagellar lengths of the *flgO* and *flgP* mutants in the $\Delta motX$ background were similar to the WT flagellar lengths (4.5 μm). Interestingly, the $\Delta motX$ mutation did not restore the flagellar defect of the *flgOP* mutant. These results suggest that the loss of flagellar rotation is sufficient to restore the length of the flagellum in the absence of either FlgO or FlgP. However, the flagellar length defect that results in the absence of both FlgO and FlgP cannot be overcome by inhibiting the rotation of the flagellum, further suggesting that *flgO* and *flgP* may function similarly to influence motility. Taken together, these data indicate that FlgO and FlgP are required for the assembly of a stable flagellum.

FlgO and FlgP are required for flagellar stability. Yoon and Mekalanos recently showed that the sheathed flagellum of *V. cholerae* is able to withstand filament dissociation at high temperatures and low pHs, unlike the unsheathed flagellum of *S. enterica* serovar Typhimurium (36). We hypothesized that if the flagella of the *flgO* and *flgP* mutants were indeed unstable, then we would expect the flagella isolated from these strains to dissociate more readily under such harsh conditions. Flagellar preparations kept at room temperature (no treatment) or treated at 50°C for 10 min showed no signs of filament dissociation (Fig. 4A). However, when the flagella were incubated at 80°C for 10 min, the *flgO*, *flgP*, and *flgOP* mutant flagella began to dissociate, whereas the WT flagella remained intact. This suggested that the sheath may be responsible for the fragile state of the flagella. However, no differences were observed between the mutant and WT flagella when treated with buffers ranging from pH 7 to pH 3 (Fig. 4B). No dissociation occurred at pH 7 or pH 5; however, at pH 3, there was flagellar dissociation of all the strains. In addition, we observed different migration patterns between the WT and mutant flagellin.

FIG. 4. The $\Delta flgO$ and the $\Delta flgP$ strains produce fragile flagella. (A) O395, $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ crude flagellar fractions were kept for 10 min at room temperature (no treatment) or incubated at 50°C or 80°C. Treated and control samples were run on 4 to 20% Tris-glycine precast gels under native conditions. Filament dissociation was determined by immunoblot analysis with anti-flagellin antibody. (B) O395, $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ flagellar fractions were treated for 10 min at room temperature with equal volumes of flagella and 50 mM citrate buffers at pH 7, 5, and 3. Treated samples were run on 4 to 20% Tris-glycine precast gels under native conditions. Filament dissociation was determined by immunoblot analysis with anti-flagellin antibody. Arrows indicate migration differences between WT and mutant flagellin.

These results indicate that the flagella produced by the $flgO$ and $flgP$ mutants are less stable than the WT but probably not strictly due to any sheath defect.

FlgO localizes to the OM and does not require FlgP for proper localization. Because FlgP was identified to be an OMP in *V. cholerae* and *C. jejuni*, we were interested in determining the localization of FlgO (25, 33). Subcellular fractionation was used to identify the OM localization of the FlgO-His₆ protein (Fig. 5A). An anti-His immunoblot revealed that the majority of the FlgO protein resides in the OM when FlgO is expressed under an inducible P_{BAD} promoter. To ensure the purity of the inner membrane (IM) and OM fractions, the fractionated samples were probed with anti-OmpA, a known OMP, and anti-ToxR, an established IM protein (IMP) (6, 24). As expected, OmpA was detected only in the OM fraction and the majority of ToxR was located in the IM, indicating that the cellular fractions obtained were not contaminated. As expected, the anti-His antibody was specific, as no protein was detected when an empty vector was expressed in the same $\Delta flgO$ strain (Fig. 5B). Additionally, when either FlgO-His₆ or FlgP-His₆ was expressed in the $flgOP$ mutant, we found that OM localization was not dependent on the other protein (Fig. 5C and

FIG. 5. FlgO localizes to the OM. Subcellular fractionation was carried out on the $\Delta flgO$ + pFlgO-His₆ (A), $\Delta flgO$ + vector (B), $\Delta flgOP$ + pFlgO-His₆ (C), and $\Delta flgOP$ + pFlgP-His₆ (D) strains. The cytoplasmic (lane C), periplasmic (lane P), IM (lane I), and OM (lane O) samples were separated by SDS-PAGE, followed by either anti-His, anti-OmpA, or anti-ToxR immunoblotting.

D). These data demonstrate that FlgO and FlgP do not require each other for proper OM localization.

FlgO and FlgP do not localize to either pole. Because of the role of FlgO and FlgP in motility and their effect on the flagellum, we hypothesized that these proteins might localize to the flagellar pole or to the flagellar sheath. Thin-sectioned cells expressing either FlgO-His₆ or FlgP-His₆ were immunogold labeled in order to localize each protein (Fig. 6). Both FlgO-His₆ and FlgP-His₆ were observed to localize throughout the membrane and were not enriched at either pole (Fig. 6A, panels i and ii). As expected, the vector controls did not result in protein detection via immunogold labeling (Fig. 6A, panels iii and iv). These results suggest that FlgO and FlgP localize throughout the OM and do not preferentially localize to the flagellar pole.

One concern about using the P_{BAD} promoter in localization experiments was the possibility of improper localization in the OM due to overexpression of the protein. To address the influence that expression levels have on localization, a strain that expresses FlgO-His₆ from its native putative promoter was constructed (the $\Delta flgO$ + $flgOp$ FlgO-His₆ strain). Although FlgO-His₆ expressed from its native promoter was not detectable via Western blot analysis, it fully complemented the motility phenotype, suggesting that the endogenous level of FlgO protein in the cell is low (data not shown). Therefore, due to the low level of protein present, the detection of immunogold particles identifying the $flgOp$ -FlgO-His₆ protein in thin-section preparations required the observation of a large number of specimens. The majority of the $flgOp$ FlgO-His₆ protein detected in the complemented strain (the $\Delta flgO$ + $flgOp$ FlgO-His₆ strain) was not localized to either pole (Fig. 6B, panels i and ii; Fig. 6C). A gold particle was observed at the pole opposite the flagellum

FIG. 6. FlgO and FlgP do not localize to either pole. (A) Thin-section immunoelectron microscopy was utilized to determine where FlgO and FlgP localized in the OM of the $\Delta flgO$ + pFlgO-His₆ (panel i) and $\Delta flgP$ + pFlgP-His₆ (panel ii) complemented strains compared to the vector-only controls. Panel iii, $\Delta flgO$ + vector control strain; panel iv, $\Delta flgP$ + vector control strain. An arrow indicates the polar flagellum. (B) Thin-section immunoelectron micrographs of the $\Delta flgO$ mutant expressing FlgO-His₆ under the control of its native putative *flgO* promoter (panels i and ii), compared to the vector control strains (panels iii and iv). Black arrows indicate gold particles, and the white arrow signifies the flagellar pole. All electron micrographs are at a magnification of $\times 20,000$. (C) Graphical representation of polar versus nonpolar (lateral) localization of *flgOp*FlgO-His₆ protein. A total of 50 cells were counted per strain. Asterisks indicate statistical comparisons (*t* test) between the nonpolar (lateral) and indicated regions (pole or nonspecific). ***, $P < 0.0001$; **, $P < 0.005$. NSB, nonspecific binding.

(Fig. 6B, panel ii), suggesting that the polar particles detected may not necessarily indicate localization at the flagellar pole. As expected, the FlgO protein was not detected at the pole or in the nonpolar membrane area in the vector-

only control (Fig. 6B, panels iii and iv; Fig. 6C). Taken together, these results suggest that FlgO localizes throughout the OM and does not specifically localize to the flagellar pole.

FIG. 7. FlgO and FlgP associate predominately with the bacterial cell and not the flagellum. Crude flagellar fractions were isolated from the O395, $\Delta flgO$ + vector, $\Delta flgO$ + pFlgO-His₆, $\Delta flgP$ + vector, $\Delta flgP$ + pFlgP-His₆, and $\Delta flaA$ (control) strains. Samples were separated by SDS-PAGE analysis followed by immunoblot analyses. Rows: A, anti-flagellin; B, anti-OmpS; C, anti-OmpU; D, anti-OmpW; E, anti-His. F, flagellar fraction; C, cellular fraction.

FlgO and FlgP are not associated with the flagellar sheath.

As a result of the thin-sectioning process, specimens yield very few cells with intact flagella. Consequently, we could not determine whether FlgO and FlgP localize to the sheath. Since the fragile state of the $\Delta flgO$ and $\Delta flgP$ flagella could be due to defects in the sheath or the filament, we next investigated whether FlgO and FlgP associated with the flagellum as a means to understand the role of FlgO and FlgP in filament stability. Crude flagellar fractions were isolated and analyzed by anti-flagellin, anti-OMPs (OmpS, OmpU, and OmpW), and anti-His immunoblotting (Fig. 7). As expected, more flagellin was detected in the flagellar fraction than in the cellular fraction for each of the WT and mutant strains (Fig. 7A). As a control, no flagellin was detected in either fraction of the aflagellated $\Delta flaA$ strain. While the OMPs were equally distributed in the cell and flagellar fractions (Fig. 7B to D), the anti-His immunoblot revealed that the majority of both the FlgO and FlgP proteins were associated with the cell, with only a slight association present in the flagellar fraction (Fig. 7E). All in all, these data suggest that although FlgO and FlgP are not part of the flagellum (Fig. 7), they do play a role in maintaining the integrity of the flagellum (Fig. 3 and 4).

FlgO plays a role in colonization. Motility plays an important role in colonization of the intestinal epithelium by *V. cholerae* (9, 12, 22). Recently, FlgP was shown to play a significant role in the intestinal colonization of the infant mouse (25). Therefore, we hypothesized that an $flgO$ mutant would also play a similar role in virulence. To determine the potential role of FlgO in colonization, the $\Delta flgO$ mutant and the $\Delta flgOP$ double mutant were assayed using the infant mouse model of cholera in which an in vivo competitive index was determined and compared to those of the $flgP$ and $flaA$ deletion mutants. We observed that the $flaA$ mutant displayed a sixfold decrease in colonization compared to the WT strain (Fig. 8A). The $flgO$ mutant also displayed a sixfold reduction in colonization, whereas the $flgOP$ mutant revealed a slightly greater decrease in its ability to colonize the infant mouse intestine. The $flgO$, $flgP$, and $flgOP$ mutants showed reduced colonization phenotypes similar to that of the $flaA$ mutant. These data suggest that FlgO also plays a role in colonization, although not as great a role as FlgP.

FlgO and FlgP are involved in epithelial cell attachment.

Because of the roles played by FlgO and FlgP in colonization, we next were interested in determining whether FlgO and FlgP are important for attachment. Also, due to the fact that motility is important for colonization and attachment, as it promotes association between the bacterial and epithelial cells, it has yet to be conclusively determined whether the flagellum contains adhesive properties (1, 9, 14, 29). To address whether the flagellum functions in adherence (contains adhesive properties) or is solely the propeller by which the cell comes in contact with the intestinal epithelium, we carried out a competitive attachment assay. The attachment assay evaluates the ability of a strain to adhere to epithelial cells in vitro. Compared to the WT strain, the nonmotile, aflagellate $flaA$ mutant displayed a threefold decrease in attachment, whereas the flagellated, nonmotile $motX$ mutant displayed a sixfold decrease in attachment (Fig. 8B). These data demonstrate that it is in fact motility, and not the adhesive ability of a flagellum, that is important for adherence. The $flgO$ mutant showed a threefold reduction in attachment compared to the WT strain, and the $flgP$ mutant displayed a similar decrease (fourfold) in attachment. The $flgOP$ mutant attachment defect was the same as

FIG. 8. The $\Delta flgO$ and $\Delta flgP$ mutants display a defect in colonization and epithelial cell adherence. (A) *V. cholerae* strain O395 and the $\Delta flaA$, $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ mutants were competed against an O395 $\Delta lacZ$ strain (KSK258), using four to seven mice per group. (B) *V. cholerae* strain O395 and the $\Delta motX$ (nonmotile, flagellate), $\Delta flaA$ (nonmotile, aflagellate), $\Delta flgO$, $\Delta flgP$, and $\Delta flgOP$ mutants were competed against an O395 $\Delta lacZ$ strain (KSK258) for in vitro attachment to HT-29 epithelial cells. Brackets indicate statistical comparisons (*t* tests) between specific data sets; otherwise asterisks indicate comparisons between the WT and the indicated strain. ***, $P < 0.0001$; **, $P < 0.005$; *, $P < 0.05$.

that of an *flgP* mutant, suggesting that FlgO and FlgP function in a common pathway. These data suggest that proper motility is crucial for attachment. Furthermore, FlgO and FlgP do not function as attachment factors per se but rather function to promote proper motility.

DISCUSSION

The purpose of this study was to identify novel factors that mediate colonization in *V. cholerae*. We have shown here that two proteins, FlgO and FlgP, appear to influence colonization by playing an important role in the stability of the flagellum. Analysis of microarray data from this study as well as data from the study presented by Morris et al. has determined that the alternate sigma factor RpoN (σ^{54}) and the class III flagellar gene activator FlrC positively regulate the *flgOP* operon (25). Morris et al. established that *flgP* is necessary for colonization by *V. cholerae*. Our studies confirm that *flgP* is involved in intestinal colonization as well as epithelial cell attachment. In addition, we have demonstrated that the adjacent gene, *flgO*, also plays an important role in motility, attachment, and colonization.

Here we report that the role of FlgO and FlgP in motility is to maintain the integrity of the flagellum. The report by Morris et al. determined that the *flgP* mutant is nonmotile; however, we have found *flgP* to be slightly motile. Despite the "occasional motile cell" that could be detected within the *flgP* mutant population by Morris et al., which is consistent with our findings, the authors did not further investigate this matter (25). We, too, noticed a small population of motile Δ *flgP* cells and therefore quantified the zones of motility as well as the percentage of motile cells within each of the *flgO*, *flgP*, and WT populations to determine the full extent of the motility defects displayed by these mutants. Based on our data, it appears that both the *flgO* and *flgP* mutants are motile; however, the motility is significantly reduced.

Morris et al. additionally report rough and perturbed flagella produced by the *flgP* mutant (25). On the contrary, we observed flagella that were smooth and displayed normal sine wave appearances for the mutant strains, similar to the WT strain. One possible cause of these conflicting observations could be the use of different negative stains. In this study we used 0.5% PTA, whereas Morris et al. used 7% uranyl acetate (UA) (25). UA and PTA are two widely used reagents for negatively staining samples for the purpose of transmission electron microscopy. One concern with using UA to negatively stain samples is the inherently low pH of the solution (11). A 1 to 2% aqueous solution of UA has a pH of 4.5 and cannot be buffered due to rapid precipitation of UA upon the addition of alkali (above pH 5.0). On the other hand, PTA can be adjusted to a more neutral pH of 6.5 for staining purposes. It was recently demonstrated that the flagella of *V. cholerae* begin to dissociate at pH 3.6, with some dissociation at pH 4.0 (36). Therefore, it is possible that the observations made by Morris et al. are due to the effect that a high concentration of UA would have on the flagellum or sheath (25). Supporting data by Sommerlad and Hendrixson showed that in the case of *C. jejuni*, the Δ *flgP* flagella appeared normal when stained with PTA (33). Likewise, Cameron et al. recently reported a normal flagellar appearance for the *flgP* mutant of the El Tor biotype

strain C6706 (5). Therefore, we conclude that the Δ *flgO* and Δ *flgP* flagella are smooth and maintain a sinusoidal appearance.

Although the appearance of the filaments appeared normal, we did observe truncated flagella in the case of the *flgO*, *flgP*, and *flgOP* mutant strains. Measurements of the *flgO*, *flgP*, and *flgOP* mutant flagella resulted in a wide distribution of lengths, ranging from no visible flagella to WT-length flagella. These observations suggest either that filament assembly is randomly interrupted or that the filament is more susceptible to breakage. Since the mutants are capable of producing normal flagella, we are in favor of the latter hypothesis. We tested the idea that the flagella of the *flgO* and *flgP* mutants were more susceptible to breakage by inhibiting the rotation of the flagellum. Flagellar rotation generates and consequently exerts forces that act upon the flagellum, which could theoretically cause a fragile flagellum to break more readily. Therefore, the use of a *motX* mutant, which is flagellated yet nonmotile, allowed us to determine whether the *flgO* and *flgP* flagella are more susceptible to breakage. Indeed, by introducing a *motX* mutation into the Δ *flgO* and Δ *flgP* background strains, we were able to restore the mutant flagella to WT lengths. This experiment implies that the flagella produced by the *flgO* and Δ *flgP* mutants are fragile and more susceptible to breakage than those of the WT. This also led us to believe that the percentage of nonflagellated cells observed in the Δ *flgO* and Δ *flgP* populations is probably the result of breakage at the base of the filament rather than lack of production of a flagellum. We propose that the shorter of the truncated flagella are not capable of further propelling the cell, thus resulting in the reduction of overall motility.

Yoon and Mekalanos recently demonstrated that the sheathed flagellum of *V. cholerae* is less susceptible to filament dissociation in the presence of high temperature or low pH than is the unsheathed flagellum of *Salmonella* serovar Typhimurium (36). To provide more evidence that the flagella of the *flgO* and *flgP* mutants are unstable, we subjected crude flagellar fractions to heat and pH treatments. We hypothesized that if the flagella of the Δ *flgO* and Δ *flgP* strains were more susceptible to breakage, then perhaps they would dissociate more readily than the WT flagella under the same harsh conditions. We found that high temperature caused the mutant flagella to dissociate more than the WT flagella, which suggests one of two things. First, the sheath of the mutant flagellum may be defective, thus allowing dissociation. Second, perhaps the sheath is normal but the rate of dissociation is higher for a shorter flagellum (Δ *flgO*, Δ *flgP*, and Δ *flgOP* mutants) than for an intact flagellum (WT) at high temperatures. However, in this case it is difficult to determine whether the flagellar instability is due to the sheath or filament.

The flagella treated with low pH did not show any differences in dissociation. All flagella dissociated at pH 3, which is consistent with the findings of Yoon et al. (36). An intriguing feature that we reproducibly observed was a distinct difference between the migration of the mutant flagellin and that of the WT flagellin under native conditions. In an attempt to identify these differences in the flagella/sheath, subsequent SDS-PAGE analysis of the WT, *flgO*, *flgP*, and *flgOP* flagellar and OM fractions was carried out and revealed a few differences in the resulting protein profiles, suggesting that the filament makeup

is somehow altered in these mutants; however, the profiles were not robust enough to make any precise conclusions (data not shown). Taken together, these results strongly suggest a filament defect. It is possible that the WT flagella are broken down into flagellin monomers while the mutant flagella (i) are not completely broken down, (ii) have some other protein or lipid moiety associated with the flagellin, or (iii) perhaps are made up of altered flagellin. If indeed flagellin is changed in some way in the mutants, then the flagellin would migrate differently under native conditions and the altered flagellin would explain why the mutant flagella are unstable. In other words, if the flagellin monomers are unable to properly polymerize, then the filament would be easily broken. Although we hypothesize that FlgO and FlgP function in proper filament assembly, we cannot ignore the possibility that these OMPs are involved in sheath stability. It is unclear whether the sheath is an extension of the OM. If this is true, then we are left with the possibility that in the absence of FlgO and/or FlgP, the sheath may be fragile, thus rendering the filament unstable.

Because of the effects that FlgO and FlgP had on flagellar stability, we were certain that we would find FlgO and FlgP to be associated with the flagellum. However, to our surprise, FlgO localization studies revealed that not only is FlgO an OMP but FlgO and FlgP can be found throughout the membrane and not enriched at either pole. In addition, we determined that FlgO and FlgP associate more readily with the cellular fraction than the flagellar fraction, suggesting that their role in flagellar stability may be indirect.

The colonization defects of the *flgO* and *flgP* mutants correlate with their motility defects. For example, the Δ *flgO* strain is more motile than the Δ *flgP* strain, and as such, it is better able to colonize. Additionally, for decades many have questioned whether colonization required motility or if it was the mere presence of a flagellum that was necessary (1, 9, 14, 22, 29). Due to the lack of molecular tools, it was not until recently that Lee et al. conclusively determined that a functional flagellum is absolutely required for colonization (22). Knowing this, we argue that because the *flgO* and *flgP* mutants are motile, they should colonize better than the Δ *flaA* strain. This holds true for the *flgO* but not the *flgP* mutant. In fact, the Δ *flgP* and Δ *flgOP* strains are significantly more defective than the *flaA* mutants in colonization. This suggests that the defect in colonization of the *flgP* mutant is separate from its motility defect. Therefore, it is possible that FlgP may also function as a colonization factor, which is consistent with the findings of Morris et al. (25). Morris et al. further hypothesize that FlgP may function as an adhesin due to the decreased competitive index of the *flgP* mutant, relative to those of other nonmotile strains (25). Based on our attachment assay results, we found the adherence index of the Δ *flgP* strain to be relatively similar to those of the *motX* and *flaA* mutants. Since the *flgP* mutant is largely defective in motility, it makes sense that its level of adherence is comparable to those of nonmotile mutants. If FlgP were functioning as an attachment factor, then we would expect to observe a significant decrease in attachment compared to the nonmotile mutants, as is seen for colonization. However, this is not the case. These data indicate that the little motility retained by the Δ *flgO* and Δ *flgP* strains is not capable of increasing their chances of coming into contact with the epithelial cells compared to those of the nonmotile strains. In

addition, since the *flaA* and *motX* mutants behaved similarly in attachment, we conclude that the *V. cholerae* flagellum does not contain adhesive properties and that a functional flagellum is absolutely required for attachment.

It remains unknown how these two proteins function within the OM to contribute to the production of a stable flagellum. We hypothesize that FlgO and FlgP affect motility in an indirect manner. Morris et al. showed that a strain expressing FlgP with a C18G mutation, which localizes to the IM rather than the OM, remained motile but was defective for colonization (25). It is important to note that OM lipoproteins are first anchored to the periplasmic side of the IM before being transported to the OM (26). Since localization of FlgP to either membrane is sufficient for proper motility, it is likely that FlgP's role in motility is carried out in the periplasm. We believe that FlgO and FlgP interact with another protein(s) that in turn interacts with a known flagellar protein(s) as a means to influence flagellar stability. Further studies are under way to identify FlgO and FlgP interacting proteins to better understand their role in flagellar stability.

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REFERENCES

1. Attridge, S. R., and D. Rowley. 1983. The role of the flagellum in the adherence of *Vibrio cholerae*. J. Infect. Dis. **147**:864–872.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, New York, NY.
3. Benítez, J. A., R. G. Spelbrink, A. Silva, T. E. Phillips, C. M. Stanley, M. Boesman-Finkelstein, and R. A. Finkelstein. 1997. Adherence of *Vibrio cholerae* to cultured differentiated human intestinal cells: an in vitro colonization model. Infect. Immun. **65**:3474–3477.
4. Butler, S. M., and A. Camilli. 2004. Both chemotaxis and net motility greatly influence the infectivity of *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **101**:5018–5023.
5. Cameron, D. E., J. M. Urbach, and J. J. Mekalanos. 2008. A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **105**:8736–8741.
6. Chai, T. J., and J. Foulds. 1977. Purification of protein A, an outer membrane component missing in *Escherichia coli* K-12 *ompA* mutants. Biochim. Biophys. Acta **493**:210–215.
7. Fuerst, J. A., and J. W. Perry. 1988. Demonstration of lipopolysaccharide on sheathed flagella of *Vibrio cholerae* O:1 by protein A-gold immunoelectron microscopy. J. Bacteriol. **170**:1488–1494.
8. Gosink, K. K., and C. C. Häse. 2000. Requirements for conversion of the Na⁺-driven flagellar motor of *Vibrio cholerae* to the H⁺-driven motor of *Escherichia coli*. J. Bacteriol. **182**:4234–4240.
9. Guentzel, M. N., and L. J. Berry. 1975. Motility as a virulence factor for *Vibrio cholerae*. Infect. Immun. **11**:890–897.
10. Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177**:4121–4130.
11. Harris, J. R. 1997. Negative staining and cryoelectron microscopy. p. 21–25. RMS microbiology handbook. BIOS Scientific Publishers Limited, Oxford, United Kingdom.
12. Häse, C. C. 2001. Analysis of the role of flagellar activity in virulence gene expression in *Vibrio cholerae*. Microbiology **147**:831–837.
13. Hranitzky, K. W., A. Mulholland, A. D. Larson, E. R. Eubanks, and L. T. Hart. 1980. Characterization of a flagellar sheath protein of *Vibrio cholerae*. Infect. Immun. **27**:597–603.
14. Jones, G. W., G. D. Abrams, and R. Freter. 1976. Adhesive properties of

- Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immun. **14**:232–239.
15. Kaur, R., K. L. Dikshit, and M. Raje. 2002. Optimization of immunogold labeling TEM: an ELISA-based method for evaluation of blocking agents for quantitative detection of antigen. J. Histochem. Cytochem. **50**:863–873.
 16. Kazmierczak, M. J., M. Wiedmann, and K. J. Boor. 2005. Alternative sigma factors and their roles in bacterial virulence. Microbiol. Mol. Biol. Rev. **69**:527–543.
 17. Kirn, T. J., B. A. Jude, and R. K. Taylor. 2005. A colonization factor links *Vibrio cholerae* environmental survival and human infection. Nature **438**: 863–866.
 18. Kirn, T. J., and R. K. Taylor. 2005. TcpF is a soluble colonization factor and protective antigen secreted by El Tor and classical O1 and O139 *Vibrio cholerae* serogroups. Infect. Immun. **73**:4461–4470.
 19. Klose, K. E., and J. J. Mekalanos. 1998. Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. Mol. Microbiol. **28**:501–520.
 20. Kojima, S., K. Yamamoto, I. Kawagishi, and M. Homma. 1999. The polar flagellar motor of *Vibrio cholerae* is driven by an Na⁺ motive force. J. Bacteriol. **181**:1927–1930.
 21. Kovacikova, G., W. Lin, and K. Skorupski. 2005. Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in *Vibrio cholerae*. Mol. Microbiol. **57**:420–433.
 22. Lee, S. H., S. M. Butler, and A. Camilli. 2001. Selection for *in vivo* regulators of bacterial virulence. Proc. Natl. Acad. Sci. USA **98**:6889–6894.
 23. Maniatis, T., E. F. Fritsch, and J. Sambrook (ed.). 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 24. Miller, V. L., R. K. Taylor, and J. J. Mekalanos. 1987. Cholera toxin transcriptional activator *toxR* is a transmembrane DNA binding protein. Cell **48**:271–279.
 25. Morris, D. C., F. Peng, J. R. Barker, and K. E. Klose. 2008. Lipidation of an FlrC-dependent protein is required for enhanced intestinal colonization by *Vibrio cholerae*. J. Bacteriol. **190**:231–239.
 26. Narita, S., S. Matsuyama, and H. Tokuda. 2004. Lipoprotein trafficking in *Escherichia coli*. Arch. Microbiol. **182**:1–6.
 27. Priefer, U. B., R. Simon, and A. Pühler. 1985. Extension of the host range of *Escherichia coli* vectors by incorporation of RSF1010 replication and mobilization functions. J. Bacteriol. **163**:324–330.
 28. Prouty, M. G., N. E. Correa, and K. E. Klose. 2001. The novel sigma54- and sigma28-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. Mol. Microbiol. **39**:1595–1609.
 29. Richardson, K. 1991. Roles of motility and flagellar structure in pathogenicity of *Vibrio cholerae*: analysis of motility mutants in three animal models. Infect. Immun. **59**:2727–2736.
 30. Sen, A., R. K. Nandi, and A. N. Ghosh. 2005. Ion-swimming speed variation of *Vibrio cholerae* cells. J. Biosci. **30**:465–467.
 31. Sjoblad, R. D., C. W. Emala, and R. N. Doetsch. 1983. Bacterial flagellar sheaths: structures in search of a function. Cell Motil. **3**:93–103.
 32. Skorupski, K., and R. K. Taylor. 1996. Positive selection vectors for allelic exchange. Gene **169**:47–52.
 33. Sommerlad, S. M., and D. R. Hendrixson. 2007. Analysis of the roles of FlgP and FlgQ in flagellar motility of *Campylobacter jejuni*. J. Bacteriol. **189**:179–186.
 34. Taylor, R. K., T. J. Kirn, M. D. Meeks, T. K. Wade, and W. F. Wade. 2004. A *Vibrio cholerae* classical TcpA amino acid sequence induces protective antibody that binds an area hypothesized to be important for toxin-coregulated pilus structure. Infect. Immun. **72**:6050–6060.
 35. Tripathi, S. A., and R. K. Taylor. 2007. Membrane association and multimerization of TcpT, the cognate ATPase ortholog of the *Vibrio cholerae* toxin-coregulated-pilus biogenesis apparatus. J. Bacteriol. **189**:4401–4409.
 36. Yoon, S. S., and J. J. Mekalanos. 2008. Decreased potency of the *Vibrio cholerae* sheathed flagellum to trigger host innate immunity. Infect. Immun. **76**:1282–1288.