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Decreased Potency of the *Vibrio cholerae* Sheathed Flagellum To Trigger Host Innate Immunity▽

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Vibrio cholerae **is a monoflagellated gram-negative bacterium that causes the severe diarrheal disease cholera. In contrast to *Salmonella enterica* serovar Typhimurium infection, which is accompanied by both acute diarrhea and high-level inflammation, *V. cholerae* infection is largely noninflammatory in human hosts. Bacterial flagella are composed of flagellin, a highly conserved protein that is also a target of the innate immune response. Because the *V. cholerae* flagellum is covered by a sheath, we hypothesized that it might be less prone to activation of the innate immune response than nonsheathed flagella, such as those produced by *Salmonella* serovar Typhimurium. Indeed, compared with *Salmonella* serovar Typhimurium flagella, *V. cholerae* flagella demonstrated significantly reduced NF-κB activation in A549 human pulmonary epithelial cells. However, *V. cholerae* flagellin monomers, FlaD and FlaC, were almost equally potent with purified FliC, a monomer derived from *Salmonella* serovar Typhimurium flagella, in NF-κB activation. Heat- and acid-induced dissociation assays showed that *Salmonella* serovar Typhimurium flagella disassembled far more readily into monomeric flagellins than *V. cholerae* flagella, suggesting that the differential levels of NF-κB activation by *V. cholerae* and *Salmonella* serovar Typhimurium flagella are likely attributable to the difference in their flagellin shedding. Our results suggest that monomer dissociation of *V. cholerae* flagella is suppressed likely due to the presence of the sheath and that this unique structural feature of *V. cholerae* flagella may have evolved as a strategy to evade flagellin-triggered host innate immune responses in various host species.**

Pathogenic bacteria utilize numerous mechanisms to colonize host surfaces and cells. Among these, flagellum-based motility is one of the more common mechanisms used by motile organisms to move toward preferred sites for colonization typically in the earliest stages of the infection process. Thus, bacterial flagella have been considered an important virulence determinant and flagellum-deficient mutants of several pathogens have been shown to be avirulent (4, 18, 28, 38, 45). All bacterial flagella are helical macromolecular structures composed predominantly of a single highly conserved protein called flagellin. This protein is recognized by the host innate immune system, the body's first line of defense against microbial infection (40, 43). The innate response to microbial invaders involves the recognition of conserved microbial products, termed pathogen-associated molecular patterns, by specific host receptors (2, 30). Germ line-encoded Toll-like receptors (TLRs) are the best characterized of these receptors. When surface-exposed TLRs bind pathogen-associated molecular patterns, signal transduction events are triggered that lead to proinflammatory cytokine production (1, 31, 36). The inflammatory reactions thus induced can lead to active clearance of pathogens as well as an enhancement of the adaptive immune response.

The discovery of TLR5 as a receptor for the bacterial protein flagellin was somewhat surprising (16), because (i) host innate immune systems were proposed to detect invariable structural products of microorganisms and (ii) there was known to exist a degree of variability among flagellins from different bacterial species. However, subsequent studies revealed that TLR5 recognizes a highly conserved dipeptide of flagellin that is essential for both flagellum assembly and motility in the *Gammaproteobacteria* (8, 29, 40). This observation was further confirmed by recent reports that the *Alphaproteobacteria* and *Epsilonproteobacteria*, whose flagellins differ in amino acid sequences in the conserved N-terminal region from those of the *Gammaproteobacteria*, can evade TLR5 recognition (3, 22).

Mounting evidence indicates that bacterial flagellins recognized by TLR5 stimulate the significant portions of intestinal inflammation (13, 34, 44). For example, flagellins were found to be dominant antigens that mediate the abnormally high steady-state intestinal inflammation in Crohn's disease (15). In addition, flagellins have been demonstrated to act as efficient vaccine adjuvants (17, 21). The elimination of the residual intestinal inflammatory-like "reactogenicity" associated with live attenuated cholera vaccines has been reported to correlate with a loss of the flagellum from two different vaccine candidates (7, 19).

Vibrio cholerae is a highly motile, gram-negative bacterium that causes a severe life-threatening diarrheal disease, cholera (49). *V. cholerae* flagellum is unique in that it is covered by a sheath. While the sheath appears microscopically to be an extension of the outer membrane, the components that make up the sheath are not clearly defined at the molecular level. Immunogold staining with monoclonal antibody has detected lipopolysaccharide O-side chains exposed on the outer surface of the flagellum sheath (10). Unidentified membrane proteins

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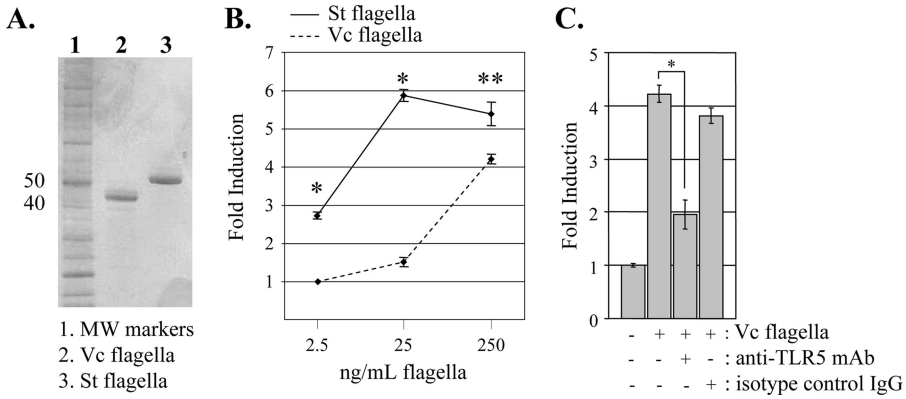

FIG. 1. NF-κB activation by purified flagella of *V. cholerae* (Vc) and *Salmonella* serovar Typhimurium (St). (A) SDS-PAGE analysis of purified flagella. One microgram of each flagellum, purified according to the procedures described in Materials and Methods, was separated in 4 to 12% SDS-PAGE. MW, molecular weight (in thousands). (B) NF-κB activation in response to flagella. Reporter activities were given as "induction (fold)," which was calculated by dividing the average activity of flagellum-stimulated A549 cells by the average activity of control phosphate-buffered saline-treated cells. $n = 8$ in each treatment, and the mean ± standard error of the mean is presented in each data point. *, $P < 0.001$; **, $P < 0.01$ versus treatment with *V. cholerae* flagella. (C) NF-κB activation by 250 ng/ml *V. cholerae* flagella in the presence of anti-TLR5 antibody or isotype control IgG. A total of 10 μg/ml of antibody was used in each treatment ($n = 8$). *, $P < 0.01$ for NF-κB induction in the absence versus the presence of anti-TLR5 antibody. mAb, monoclonal antibody.

also have been shown to copurify with *V. cholerae* flagella (26). Critically, the immunostimulatory activity of the sheathed *V. cholerae* flagella has not been determined vis-à-vis unsheathed flagella from other gram-negative species. In this study, we demonstrated that sheathed *V. cholerae* flagella are dramatically reduced in their relative potency to trigger host innate response compared to unsheathed flagella. This reduced potency appears to be associated with the stability of the sheathed structure rather than primary alterations in the flagellin amino acid sequence.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Salmonella enterica* serovar Typhimurium strain LT2 and *V. cholerae* seventh pandemic El Tor biotype strain C6706 were used in this study. Bacterial cultures were grown at 37°C in LB (10 g tryptone, 5 g NaCl, and 5 g yeast extract per liter) with shaking. The antibiotics streptomycin (200 μg/ml for *V. cholerae*), kanamycin (100 μg/ml for *V. cholerae*), and ampicillin (100 μg/ml for *Escherichia coli*) were used as required.

Purification of flagella and recombinant flagellins. For flagellum purification, bacterial cell suspension was mechanically sheared by a Tissue-Tearor (model 985370; BioSpec Products, Inc., Bartlesville, OK) for 1 min at full speed. Bacterial cells were removed by centrifugation at 10,000 rpm for 10 min. The supernatant containing flagella was further centrifuged at 210,000 × *g* for 90 min at 4°C. Flagellum pellet was resuspended in phosphate-buffered saline and then subjected to 1.37-g/ml CsCl density gradient ultracentrifugation at 100,000 × *g* for 18 h at 25°C. The next day, a flagellum band was isolated by syringe and dialyzed against water overnight. For recombinant flagellin production, the *flaD*, *flaC*, and *fliC* genes were PCR-amplified and positionally cloned into pET21b (Novagen). The resulting plasmid was then transformed into *E. coli* BL21(DE3). IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) was used to induce overexpression, and recombinant His-tagged proteins were purified using a nickel-nitrilotriacetic acid column (Qiagen, Valencia, CA). The purity of the flagella and recombinant flagellins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A549 cell culture. Human alveolar A549 epithelial cells, in which a luciferase-based NF-κB reporter construct was stably transfected (Panomics, Inc., Fremont, CA), were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/ml hygromycin at 37°C in a humidified 5% CO_2 incubator. To measure NF-κB activation, 2×10^4 cells were seeded in each well of a 96-well plate and cultured overnight. The next day, cells were treated with flagella or flagellins for 4 h. Luciferase activity was measured using a Bright-Glo luciferase assay kit (Promega). A Spectrafluor Plus plate reader (Tecan US, Research Triangle Park, NC) was used to measure the luciferase activity. In the NF-κB reporter assay with a TLR5 neutralizing antibody, anti-TLR5 goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and purified goat immunoglobulin G (IgG) (Sigma) were used. A549 cells pretreated with each antibody for 30 min were stimulated with *V. cholerae* flagella (250 ng/ml) for 4 h before luciferase assays were performed.

Two-dimensional gel electrophoresis. The Zoom IPGRunner system (Invitrogen) was used for isoelectric focusing. The separation of flagellins whose pI values are close to each other was achieved by using a pH strip with narrow pH range (pH 4.5 to 5.5; Invitrogen). After isoelectric focusing, the pH strip was cropped at both ends to match the width of the well of the 4 to 12% gel used for second-dimensional SDS-PAGE. Protein spots were visualized by silver nitrate and cut out for identification by mass spectrometry (Taplin Biological Mass Spectrometry Facility, Department of Cell Biology, Harvard Medical School).

Native gels, motility assay, and electron microscopy. Flagellum disassembly was assayed by running a native gel of heat- or acid-treated flagella. In this gel-based assay, only depolymerized flagellins that migrated into the gel were detected as a band, while intact flagellum filament remained in the loading well. For acid treatment, 50 mM sodium citrate buffers with pH values ranging from 2.8 to 5.2 were mixed with the same volume of flagellum samples for 10 min.

Statistical analysis. Data are expressed as means ± standard errors of the means. An unpaired Student's *t* test was used to analyze the data. A *P* value of <0.05 was considered statistically significant.

RESULTS

***V. cholerae* flagella are less potent in activating the NF-κB signaling cascade than *Salmonella* serovar Typhimurium flagella.** To examine the capacity of *V. cholerae* flagella to activate the innate immune response, A549 luciferase-based NF-κB reporter cells were treated with *V. cholerae* or *Salmonella* serovar Typhimurium flagella, the latter of which are prototypic bacterial flagella that have been extensively studied in the context of TLR5-mediated innate responses. *V. cholerae* and *Salmonella* serovar Typhimurium flagella were purified as described in Materials and Methods. SDS-PAGE analysis of the purified flagella clearly showed an ~51-kDa FliC, a flagellin component of *Salmonella* serovar Typhimurium flagella, and ~40-kDa flagellins derived from *V. cholerae* flagella (Fig. 1A). *V. cholerae* flagellins migrated slower than their molecular weight on SDS-PAGE. We chose A549 cells because they have

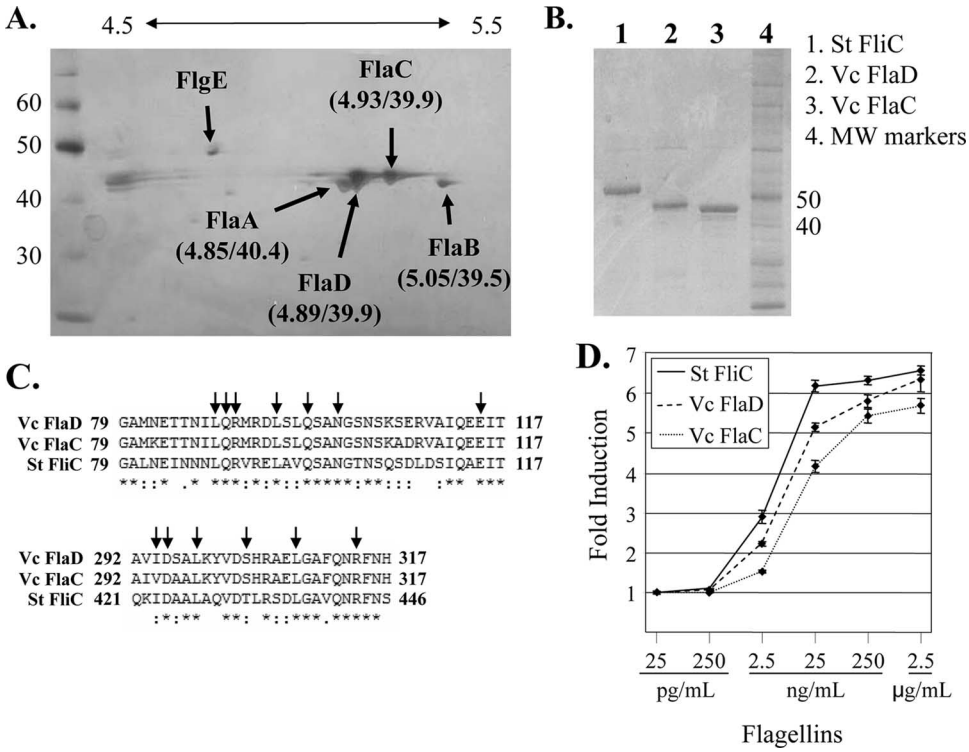

FIG. 2. Identification of predominant flagellins in *V. cholerae* (Vc) flagella and NF-κB activation by purified flagellins of *V. cholerae* and *Salmonella* serovar Typhimurium (St). (A) Two-dimensional-gel/mass-spectrometry analysis of *V. cholerae* flagella. One microgram of purified C6706 flagella was separated via a two-dimensional gel in which a narrow-ranged pH strip (4.5 to 5.5) and 4 to 12% SDS-PAGE were used for first- and second-dimensional separation, respectively. Proteins were visualized by silver staining and identified by liquid chromatography-tandem mass spectrometry. pI and MW (in thousands) values for the flagellin proteins are shown together in parentheses. (B) SDS-PAGE analysis of purified recombinant flagellins. One microgram of each flagellin was separated by 4 to 12% SDS-PAGE. MW, molecular weight (in thousands). (C) Primary sequence alignment of FlaD (VC2143), FlaC (VC2187), and FliC (AAL20871) in highly homologous N- and C-terminal regions. Black arrows indicate residues that were determined to be essential for TLR5 signaling in reference 40. Alignment was generated using the ClustalW program. (D) NF-κB activation in response to flagellins ($n = 8$). "Induction (fold)" was measured as described in the legend for Fig. 1B.

been demonstrated to respond strongly to bacterial flagella (44, 46). As shown in Fig. 1B, significantly reduced NF-κB activation was detected in cells treated with *V. cholerae* flagella, compared to those treated with *Salmonella* serovar Typhimurium flagella. In each case, NF-κB activation was dose dependent. When neutralizing anti-TLR5 antibody was added to the culture of A549 reporter cells, significantly reduced NF-κB activation was detected from the same treatment, confirming the previous reports that TLR5 is responsible for flagellin recognition (Fig. 1C).

Purified *V. cholerae* and *Salmonella* serovar Typhimurium flagellins are similar in their potency for NF-κB activation in A549 cells. It has been reported that the TLR5-mediated proinflammatory signal was stronger in cells treated with purified flagellins than in cells responding to flagellum filament (40). Therefore, we asked if decreased NF-κB activation by *V. cholerae* flagella was attributed to the reduced potency of *V. cholerae* flagellins for NF-κB activation. Since the *V. cholerae* genome encodes five different genes encoding flagellins, we first determined which flagellins are the predominant proteins in purified *V. cholerae* flagella by two-dimensional-gel/mass-spectrometry analysis. Using a narrow-range pH gradient (pH 4.5 to 5.5) for isoelectric focusing and then SDS-PAGE, flagellin components in *V. cholerae* flagella, which are indistinguishable by SDS-PAGE (Fig. 1A), were successfully separated (Fig. 2A). Our results showed that *V. cholerae* flagella are comprised of four different flagellins: FlaD, FlaC, FlaA, and FlaB. In addition, FlgE (VC2197), the flagellar hook protein, was also detected in the purified flagellum preparations.

We next examined the signal-activating potential of FlaD and FlaC relative to FliC using the same A549 cell reporter system. We selected FlaD and FlaC, because they were identified as the most abundant subunits in *V. cholerae* flagella (Fig. 2A). The three flagellin proteins were purified as six-His-tagged recombinant proteins in *E. coli*, and the purity of each protein is shown in Fig. 2B. Primary sequence alignment showed that all three flagellins are highly homologous in the first ~120 and last ~90 amino acids, which were reported to be essential for motility and form the conserved D1 domain recognized by TLR5 (40, 46). In particular, 11 out of the 13 amino acid residues that were determined to be essential for TLR5 signaling by alanine scanning mutagenesis (40) were identical in all three flagellins (Fig. 2C). The central regions of these flagellins, however, are significantly variable in both size and amino acid sequence (data not shown). When A549 cells were treated with purified flagellins, dose-dependent NF-κB activation was invariably detected in response to each of the three flagellins (Fig. 2D). NF-κB activation was slightly higher in

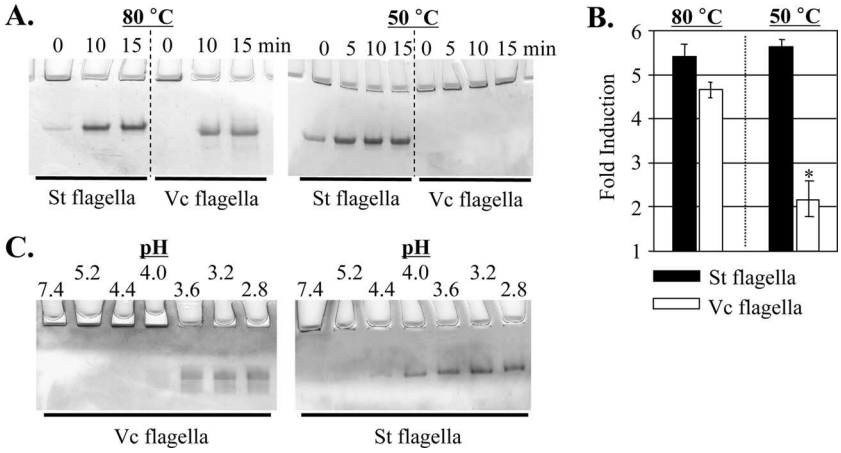

FIG. 3. Heat- and acid-induced flagellum disassembly and NF-κB activation by heat-treated flagella. (A) Native gels of heat-treated flagella. Both flagella were incubated at either 50°C or 80°C for 15 min. Samples at 5-, 10-, and 15-min time points were loaded onto the 4 to 12% gel. Results are representative of three independent experiments. St, *Salmonella* serovar Typhimurium; Vc, *V. cholerae*. (B) NF-κB activation by heat-treated flagella. Both flagella at 25-ng/ml concentrations were treated at 50°C or 80°C for 10 min and added to A549 reporter cells. Experimental conditions were identical with those described in the legend for Fig. 1B and 2D. *, $P < 0.01$ versus treatment with *Salmonella* serovar Typhimurium flagella pretreated at 50°C. (C) Native gels of acid-treated flagella. Flagella were mixed with the same volume of each citrate buffer (50 mM) with the pH value shown at the top of the gel. After 10 min, samples were loaded onto the gel. Results are representative of three independent experiments.

cells treated with FliC than in cells responding to FlaD or FlaC (Fig. 2D). This result, however, does not fully explain the significantly reduced NF-κB activation by *V. cholerae* flagella shown in Fig. 1B, suggesting that some other factor might be responsible for the differential NF-κB activation between *V. cholerae* and *Salmonella* serovar Typhimurium flagella. Most importantly, FlaD or FlaC at a concentration of 25 ng/ml induced a three- to fourfold-higher NF-κB signal than a corresponding amount of flagellum filament (*V. cholerae* flagella). In contrast, we observed comparable levels of NF-κB activation from A549 cells when treated with either *Salmonella* serovar Typhimurium flagella or a corresponding amount of FliC (Fig. 2D and 1B).

Monomer dissociation of *V. cholerae* flagellum is reduced, while *Salmonella* serovar Typhimurium flagellum dissociates into its monomer subunits readily. Since *V. cholerae* flagella are sheathed and TLR5 recognizes a conserved domain of flagellin, we next asked whether the NF-κB activating potential of *V. cholerae* flagella would be influenced by their unique sheathed structure. Since no mutants exist that specifically eliminate the sheath from *V. cholerae* flagella, we developed biochemical assays to assess the propensity of sheathed *V. cholerae* flagella to shed monomeric flagellins compared to nonsheathed *Salmonella* serovar Typhimurium flagella. The assay involved native PAGE in which flagellin monomers and filamentous flagella were easily distinguished (Fig. 3A and C). Flagellins were detected as a distinct band in the gel, while filamentous flagella did not migrate into the gel. When purified flagella were heat treated at 80°C for 15 min, both *Salmonella* serovar Typhimurium and *V. cholerae* flagella completely dissociated into their respective flagellins (Fig. 3A). Flagellins derived from *V. cholerae* flagella migrated slightly faster than *Salmonella* serovar Typhimurium flagellins, likely due to their smaller size. During incubation at 50°C, however, only *Salmonella* serovar Typhimurium flagella, not *V. cholerae* flagella, dissociated into monomers. These results indicate that *V. cholerae* flagella are physically stronger than *Salmonella* serovar Typhimurium flagella. Interestingly, even before thermal treatment, a significant portion of *Salmonella* serovar Typhimurium flagellum preparations migrated as flagellins, suggesting that monomer dissociation occurs readily for *Salmonella* serovar Typhimurium flagella. Reassembled *Salmonella* serovar Typhimurium flagellum filaments were not detected even after 30 min of cooling down to room temperature (data not shown), suggesting that monomer dissociation was not reversible. Next, A549 reporter cells were incubated with 25 ng/ml of each flagellum that was pretreated at 80°C or 50°C for 10 min. Figure 3B shows the NF-κB activation by heat-treated flagella. *Salmonella* serovar Typhimurium flagella pretreated at either temperature for 10 min elicited a high level of NF-κB activation (Fig. 3B). In contrast, a comparable NF-κB induction by *V. cholerae* flagella was detected only at 80°C, not at 50°C (Fig. 3B). These results confirmed the previous finding that the higher level of NF-κB signaling is triggered by flagellin monomers rather than by their filamentous flagella counterparts and suggested that the remarkably delayed NF-κB activation by *V. cholerae* flagella shown in Fig. 1B was due to their diminished monomer dissociation.

Finally, we also asked if these two flagella exhibit differential disassembly patterns under acidic stress. As shown in Fig. 3C, disassembly of *Salmonella* serovar Typhimurium flagella started at a pH of 4.4, while depolymerization of *V. cholerae* flagella occurred at pH 3.6 or lower. These results indicate that *V. cholerae* flagella are also more resistant to acid-induced depolymerization than *Salmonella* serovar Typhimurium flagella. While the differences observed in the thermo and acid stabilities of *V. cholerae* and *Salmonella* serovar Typhimurium flagella might reflect differences in the stability of the two different flagellar filaments per se, it is also possible that the sheath of the *V. cholerae* flagella represents a specific barrier to the

release of flagellin subunits under physical and pH stresses; alternatively, the sheath might improve the stability of flagellum filaments in the face of such stresses.

DISCUSSION

A hallmark of *V. cholerae* is the ability to cause watery diarrhea without stimulating host inflammation (11). Although this unique disease progression could be due to rapid bacterial "washout" by fluid secretion, resulting in a reduced level of bacterial contact with the intestinal epithelium, accumulating evidences suggest that *V. cholerae* infection is largely noninflammatory. The predominant virulence factor that is responsible for the severe diarrhea is cholera toxin (CT), a product of the *ctxA* and *ctxB* genes carried on transmissible phage CTXΦ (47). CT has been reported to suppress the production of proinflammatory cytokines in cultured macrophages (5) and human dendritic cells (12). In accordance with this finding, live attenuated vaccine strains that do not produce CT caused a more inflammatory diarrhea in volunteer studies (39). However, the immune suppressive mechanisms of CT are not clearly defined, and the proinflammatory activities of *V. cholerae* may be upregulated by other mechanisms in nontoxigenic mutants. In addition, the flagellum-mediated immune stimulatory effects in *V. cholerae* infection have also received considerable attention, because undesirable reactogenicity detected in multiple-toxin-deficient vaccine candidate strains (27, 42) was abrogated when flagellum-based motility was disrupted (19, 32). This suggests that *V. cholerae* flagella contribute to part of the organism's reactogenicity but are dispensable for immunogenicity. Although flagellum-mediated motility has been shown to contribute to *V. cholerae* pathogenesis (6, 35), the role of the sheathed flagellum as an immunogenic factor remains elusive. A detailed understanding of the ability of *V. cholerae* sheathed flagella to induce proinflammatory responses is therefore of practical clinical interest and needs to be investigated further. In this work, we revealed unique structural characteristics of *V. cholerae* flagella affecting their potential to stimulate host innate immune responses.

This study was initiated based on an intriguing observation that flagella derived from *V. cholerae* elicited significantly reduced TLR5-mediated NF-κB activation compared to flagella purified from *Salmonella* serovar Typhimurium. *Salmonella* species are invasive organisms that usually induce acute intestinal inflammation during their colonization (24–26). The goal of the current study was to ask whether differences in the abilities to shed flagellins might account in part for the differences seen in intestinal inflammation encountered after *V. cholerae* versus *Salmonella* serovar Typhimurium infections. Our first experiments tested the idea that flagellin monomers from *V. cholerae* and *Salmonella* serovar Typhimurium might have differential activities in NF-κB activation assays. Since *V. cholerae* possesses five different flagellin-encoding genes distributed at two different loci of the chromosome, we sought to find the relative expression level of each flagellin in the *V. cholerae* flagella. Using two-dimensional-gel/mass-spectrometry analysis, we found that (i) each of four different flagellins (FlaA, FlaB, FlaC, and FlaD) was incorporated as a part of the flagella and (ii) among them, FlaD and FlaC were most abundant (Fig. 2A). This was surprising because FlaA appeared to be the essential component, based on a previous study where the loss of FlaA was found to result in a loss of motility (20). Consistent with this finding, mutant derivatives of strain C6706 defective in the FlaB, FlaC, or FlaD genes still exhibited wild-type-level motility (data not shown). This result suggests that *V. cholerae*, as a highly motile bacterium, might utilize flagellin redundancy for some other purpose. Since *V. cholerae* FlaD and FlaC were found to be proinflammatory in cellular assays (Fig. 2D), it is conceivable that these alternative flagellins could play a proinflammatory role in the *V. cholerae*-host interaction. Consistent with this idea is the recent report that FlaD and FlaC are secreted outside the cells in the culture of *flaA*-deficient nonflagellated mutants (48). If conditions exist that downregulate FlaA, *V. cholerae* might become more proinflammatory through the secretion of FlaD and FlaC. Because the synthesis of FlaD or FlaC is not affected by the absence of FlaA, this result might also suggest that flagellum assembly depends strictly on the presence of FlaA.

In A549 reporter cells, purified FlaD or FlaC induced comparable levels of NF-κB activation as purified FliC (Fig. 2D). An analysis of sequence alignment showed that all three flagellins were 52% identical in both the first 120 and last 83 amino acids. Recognition of FliC by TLR5 was reported to involve the conserved fold of the protein, which consists of N- and C-terminal regions (3, 9, 40, 46). Especially, five residues in the region of amino acids 89 to 98 that were determined to be essential for TLR5 signaling (40, 46) were identical among these flagellins (Fig. 2C). These results imply that the flagellin monomers from these two bacteria are similar to each other, albeit different in size, in terms of the three-dimensional fold of these conserved domains and thus likely to be equivalently recognized by TLR5 and thus elicit similar levels of NF-κB activation.

Both *V. cholerae* and *Salmonella* serovar Typhimurium belong to the family of *Gammaproteobacteria*. Recently, Andersen-Nissen and colleagues reported that flagellins derived from several bacterial species in this family strongly induced TLR5 signaling (3). However, all *Vibrio* species, including *V. cholerae*, possess flagella with a distinct structural feature, i.e., the presence of a sheath around the flagellum protofilament, which is not observed in other members of *Gammaproteobacteria*. We obtained compelling evidence indicating that flagella isolated from *V. cholerae* and *Salmonella* serovar Typhimurium differ in their tendencies to dissociate into monomeric subunits (Fig. 3A and C). *V. cholerae* flagella were resistant to heat- and acid-induced depolymerization, while *Salmonella* serovar Typhimurium flagella were easily disassembled into monomeric subunits. This suggests that sheathed flagella are less fragile and thus that monomer shedding could be minimized during infection. It is also possible that the unique multiflagellin composition of *V. cholerae* flagella also contributes to their improved thermo and pH stabilities. Because flagella play a critical role in chemotaxis, it is possible that the improved stability of *Vibrio* sp. flagella may also be a reflection of the nutritionally challenging environmental niche to which members of this genus are so well adapted.

TLR5 was shown to be expressed exclusively on the basolateral surface of intestinal and gastric epithelia (14, 33, 37). This indicates that flagellins might need to be translocated

across the epithelium to activate TLR5 signaling. This might suggest that intestinal epithelial cells selectively respond to flagellins derived from invasive, not commensal (noninvasive), bacteria. Recently, Lyons and colleagues reported that flagellins of *Salmonella* serovar Typhimurium are transcytosed and delivered to basolateral TLR5 through the host vesicular trafficking system during infection (23). Interestingly, the flagellin transcytosis occurred even in a nonflagellated mutant strain that still secretes the flagellin monomers, suggesting that (i) flagellin uptake by the host epithelium is not associated with bacterial motility per se and (ii) flagellum depolymerization into flagellin monomers is the critical step for the recognition of flagellin by TLR5. In this respect, reduced monomer shedding, as observed in *V. cholerae* flagella, could serve as a novel protective mechanism for an evasion of host innate immunity.

In summary, we have shown evidence for a novel mechanism by which bacteria with sheathed flagella can potentially evade flagellin-dependent host innate immune responses. The flagella of invading pathogenic bacteria are recognized as a major target of the host innate immune system, and the intestinal epithelium is highly specialized to respond to bacterial flagellin (14, 41). Our data suggest that *V. cholerae* might utilize a more stable sheathed flagellum during intestinal colonization and thus minimize the release of flagellin and induction of proinflammatory responses that might be deleterious to this organism's infection process.

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