Identification of Proinflammatory Flagellin Proteins in Supernatants of *Vibrio cholerae* O1 by Proteomics Analysis*§

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The genome of *Vibrio cholerae* contains five flagellin genes that encode proteins (FlaA–E) of 39–41 kDa with 61–82% identity among them. Although the existing live oral attenuated vaccine strains against cholera are protective in humans, there is an intrinsic residual cytotoxic and inflammatory component associated with these candidate vaccine strains. Bacterial flagellins are known to be potent inducers of proinflammatory molecules via activation of Toll-like receptor 5. Here we found that purified flagella from wild type *V. cholerae* 395 induced significant release of interleukin (IL)-8 from cultured HT-29 human colonic epithelial cells. Furthermore we found that filtered supernatants of KKV90, a ΔflaA isogenic strain unable to produce flagella, were still able to activate production of IL-8 albeit to significantly lower levels than the wild type, suggesting that other activators of proinflammatory molecules were still present in these supernatants. A comparative proteomics analysis of secreted proteins of *V. cholerae* 395 and KKV90 identified additional proteins with potential to induce IL-8 release in HT-29 cells. Secreted proteins in the range of 30–45 kDa identified by two-dimensional electrophoresis and mass spectrometry revealed the presence of two additional flagellins, FlaC and FlaD, that appeared to be secreted 3- and 6-fold more, respectively, in the mutant compared with the wild type. Double isogenic mutants flaAC and flaAD were unable to trigger IL-8 release from HT-29 cells. In sum, we have shown that purified flagella and secreted flagellin proteins (FlaC and FlaD) are inducers of IL-8 release from epithelial cells via Toll-like receptor 5. This observation may explain, in part, the observed reactogenicity of cholera vaccine strains in humans. *Molecular & Cellular Proteomics* 5:2374–2383, 2006.

Cholera remains a devastating bacterial cause of human morbidity and mortality in some areas of the world (1). The disease is produced by *Vibrio cholerae*, a Gram-negative curved rod that colonizes the human intestine where it secretes the potent cholera toxin (CT), which ultimately stimulates cellular adenylate cyclase to cause massive intestinal fluid loss leading to profuse watery diarrhea. CT is the major *V. cholerae* virulence factor, and it is encoded by the ctxA and ctxB genes carried on the transmissible prophage CTXφ (2). *V. cholerae* produces an array of virulence factors, which are coordinately regulated by the transcriptional activator ToxR (3). In turn, ToxR activates ToxT, a second transcriptional regulator that activates the expression of CT and the toxin-coregulated pilus (TCP) (4, 5). TCP is considered the most important intestinal colonization factor of *V. cholerae* (6).

In addition to CT, the accessory cholera toxin (Ace) (7) and the zonula occludens toxin (Zot) (7, 8) were reported as potential cytotoxic factors, but these proteins were later demonstrated to be components of a filamentous bacteriophage (2). Several *in vitro* studies have shown that *V. cholerae* secretes other cytotoxic factors such as the hemagglutinin/protease (HAP), hemolysin (Hly), and repeats-in-toxin (RTX) (9–11). These cytotoxic factors may cause tissue damage by different mechanisms that could contribute to proinflammatory responses. However, only RTX mutants have been demonstrated in a murine pulmonary cholera model to show less severe pathology and decreased serum levels of proinflammatory IL-6 and murine macrophage inflammatory protein-2, suggesting that RTX participates in the severity of acute inflammatory responses (12).

Research on cholera vaccines has focused largely on oral formulations that stimulate the mucosal immune system thereby mimicking natural infection (13). Through the years, different formulations of cholera vaccines have been proposed that include formalin or heat-killed bacteria alone or in combination with CT B-subunit. As new putative virulence factors were discovered, oral live attenuated vaccines carrying single or multiple mutations in ctx, hly, hap, rtx, tcp, ace,

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The abbreviations used are: CT, cholera toxin; Fla, flagellin subunit; LB, Luria-Bertani, IL, interleukin; Hly, hemolysin; HAP, hemagglutinin/protease; RTX, repeats-in-toxin; TCP, toxin-coregulated pilus; TLR, Toll-like receptor; 2-D-GE, two-dimensional gel electrophoresis; TEM, transmission electron microscopy.
zot, acf, and cep among others were constructed and tested in animal models and in human volunteers (1, 14–20). A significant reduction in virulence and increase in the level of protection have been achieved by some of these candidate vaccine strains with some advantages over others. For example, the whole cell, B-subunit, inactivated vaccine is effective only in multiple doses to confer protection, and immunity wanes over time (21). CVD 103-HgR is a live, single dose attenuated vaccine derived from classical V. cholerae O1 that confers 82–100% protection against classical cholera but is less effective against El Tor cholera, the predominant biotype in the Indian subcontinent (22). Two live attenuated oral vaccine strains (Peru-3 and Peru-15) derived from a Peruvian strain (C6709) V. cholerae O1, biotype El Tor, have been tested in human volunteers (20). Peru-3 is a flagellated and motile strain that provided 100% protection but remained reactogenic in human volunteers. In contrast, the flagella-less, non-motile derivative Peru-15 was safe, immunogenic, and protective as it conferred 100% protection with no reactogenicity (15). These studies suggest that there are sufficient indications that in the absence of CT some strains may also cause disease or undesirable symptoms such as sporadic outbreaks of watery diarrhea, vomiting, nausea, abdominal cramps, hemorrhage, headache, tissue damage, and septicemia (23–25). Infiltration of polymorphonuclear neutrophils, elevated levels of lactoferrin, and inflammatory enterocolitis caused by CT-minus V. cholerae strains are indications of an inflammatory response (12, 26, 27). Cholera is considered a non-inflammatory secretory disease; however, these data indicate that CT-negative V. cholerae strains induce reactogenicity and a more inflammatory diarrhea rather than the non-inflammatory disease associated with CT-producing strains (12). The proinflammatory component(s) responsible for the residual reactogenicity observed with live attenuated vaccines in human volunteers remain to be identified (28). The fact that live attenuated vaccine lacking flagella are non-reactogenic suggests that the flagella may be associated with the intrinsic reactogenicity of motile vaccine strains.

Motility in V. cholerae is driven by a single polar flagellum, and it is clear that both expression of flagella and motility contribute to colonization and virulence (29–32). The genome of V. cholerae contains five flagellin genes that encode proteins (FlaA–E) of 39–41 kDa with significant identity (ranging from 61 to 82%) among them. FlaA is essential for assembly and function of the flagellum because a mutation in flaA, but not in the remaining four flaBCDE genes, abolished flagella production and motility. Although the five flagellin genes are all actively transcribed in motile strains (33), the presence of FlaB, FlaC, FlaD, and FlaE within the flagellum has not been formally demonstrated. A flagellar sheath, apparently composed of outer membrane, coats the surface of the flagellum (34). Richardson and Parker (35) reported that the sheathed flagellum of V. cholerae was composed of one flagellar protein and one outer membrane protein of 47 and 49 kDa, respectively, but these proteins were not identified biochemically. The contribution of the sheath to motility and/or masking the immune stimulatory nature of the flagellins has not been elucidated. The flagellins of many bacterial pathogens are potent inducers of proinflammatory molecules via activation of basolateral Toll-like receptor 5 (TLR5) in epithelial and phagocytic (monocytes and dendritic) cells (36, 37). The flagellin-TLR5 signaling pathway is emerging as a paradigm in the interaction of motile bacteria with eukaryotic cells, and this interaction contributes to induction of dendritic cell maturation and differentiation (38, 39). In general, bacterial monomeric flagellin elicits synthesis and release of the proinflammatory cytokines tumor necrosis factor-α, interleukins IL-8, IL-1β, IL-6, and IL-10; and γ interferon from a variety of host cells (for a review, see Ref. 40). Proinflammatory monomeric flagellin produced by Salmonella during infection of intestinal epithelial cells is synthesized and secreted de novo by the bacterium after direct sensing of host-produced lysophospholipids (41). The role of V. cholerae flagellins in activation of proinflammatory molecule expression in epithelial cells has not been explored.

In this study, we used ultrastructural, genetic, and proteomics analyses to identify V. cholerae products responsible for inducing the release of IL-8, a biomarker of inflammation, from HT-29 human colonic epithelial cells. We found that purified V. cholerae flagella alone and two flagellin subunits, FlaC and FlaD, found in cell-free supernatants are activators of IL-8 release from epithelial cells. Double isogenic mutants flaAD and flaAC were drastically reduced in their ability to trigger IL-8 release, suggesting a synergetic effect. These observations may explain the reactogenicity associated with non-CT-producing V. cholerae live oral attenuated vaccine strains.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Strains used in this study are listed in Table I. Bacterial routine cultures were prepared in Brucella broth with shaking at 37 °C unless otherwise indicated. For preparation of secreted proteins, V. cholerae strains were grown in different bacteriological media (described below). Antibiotics were added to the media as needed.

Electron Microscopy—For transmission electron microscopy (TEM), 10 µl of bacterial cultures or protein extracts were placed onto 300-mesh carbon Formvar copper grids (Ted Pella, Inc., Redding, CA) and removed after 5 min. The sample was negatively stained with 1% phosphotungstic acid (pH 7.4) for 5 min and rinsed with one drop of sterile water. The dried samples were viewed using a Philips CM12 electron microscope at 80 kV. For immunoelectron microscopy, rabbit polyclonal antibody against Escherichia coli H7 flagella or against outer membrane protein (OMP) available from a previous study (42, 43) and anti-rabbit IgG antibody conjugated to 10-nm gold particles were used at 1:10 dilutions in PBS containing 1% bovine serum albumin (42).

Preparation of Purified Flagella—V. cholerae 395 was grown on 100 Brucella agar at 37 °C and then resuspended in 100 ml of PBS. The flagella were mechanically sheared from the surface of the bacteria and separated by centrifugation at 18,000 × g. The supernatant was cleared by centrifugation at 22,000 × g, and the flagella were pelleted by ultracentrifugation at 110,000 × g and then applied onto...
Coomassie Brilliant Blue R-250 (OmniPur, EM Science, Gibbstown, NJ) and bacteria were used to prepare whole cell extracts in sample denaturation by mass spectrometry analysis (Proteomics Core Facility, College of Pharmacy, University of Arizona). The presence and purity of the flagella was assessed by TEM screening for motility in motility soft agar and for production of flagella previously (33). The mutants obtained were verified by PCR and by allelic exchange and homologous recombination as described previously (33). The mutants obtained were verified by PCR and screened for motility in motility soft agar and for production of flagella by TEM and immunoblotting. Other flagella mutants used were available from a previous study (Table I) (33).

**Construction of Isogenic Mutants—** Double isogenic mutants, KK-VAC (flaAC) and KKVD (flaAD), were constructed in *V. cholerae* 395 by allelic exchange and homologous recombination as described previously (33). The mutants obtained were verified by PCR and screened for motility in motility soft agar and for production of flagella by TEM and immunoblotting. Other flagella mutants used were available from a previous study (Table I) (33).

**Induction of IL-8 Release in HT-29 Colonic Cells—** Confuent epithelial monolayers of HT-29 cells were grown as described previously (36) and equilibrated with 1 ml of Hank's balanced salt solution plus Ca<sup>2+</sup>, Mg<sup>2+</sup>, and 10 mM HEPES, pH 7.4 (Sigma), in the lower wells and 100 µl in the upper wells and incubated for 30–45 min at 37 °C. To infect monolayers, 10 µl of a *V. cholerae* suspension (2 × 10<sup>5</sup>/ml (multiplicity of infection, ~70) were added to the apical well of the cultured monolayers and incubated for 1 h at 37 °C. Monolayers were then carefully washed of excess bacteria and placed in 300 µl of Hank's balanced salt solution in the lower (basolateral) well and incubated at 37 °C. The basolateral medium was collected 4 h later, and the concentration of IL-8 was analyzed by ELISA.

**IL-8 ELISA—** A sandwich ELISA using 96-well plates coated overnight at 4 °C with a concentration of 8 µg/ml goat anti-human IL-8 (R&D Systems, Minneapolis, MN) was performed as described previously (46). Dilutions of the supernatants were added to the wells followed by rabbit anti-human IL-8. The complex was detected with goat anti-rabbit IgG peroxidase conjugate (Sigma) and the peroxidase activity was measured using ABTS as the substrate. Absorbance was read at an optical density of 405 nm.

**Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin.**

### TABLE I

**Phenotype and genotype of *V. cholerae* strains used**

| Strain | Genotype* | Motility | OmpU | Flagella | Ref. |
|--------|-----------|----------|------|----------|------|
| 395    | flaA-Cmr | +        | +    | +        | 47   |
| KKV7   | flaA::pGP704 | +   | +    | +        | 33   |
| KKV22  | flaB1::pGP704 | +   | +    | +        | 33   |
| KKV34  | flaD1::Cmr | +        | +    | +        | 33   |
| KKV90  | flaA1::Cmr | +        | -    | -        | 33   |
| KKV171 | flaC1::pGP704 | +   | +    | +        | 33   |
| KKV172 | flaC1::pGP704, flaD1::Cmr | +   | +    | +        | 33   |
| KKV174 | flaC1::pGP704, flaD1::Cmr | +   | +    | +        | 33   |
| KKVAC  | flaA, flaC::Amp | -    | +    | -        | This study |
| KKVD   | flaA, flaD::Amp | -    | +    | -        | This study |

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Induction of IL-8 release in HT-29 colonic cells was performed according to the method of O’Farrell (45) as follows. IEF was carried out in glass tubes of 2.0-mm inner diameter using 2% pH 4–8 Ampholines (British Drug House, Gallard Schlesinger, Long Island, NY) for 9600 V-h. One microgram of an IEF standard, tropomyosin, was added to each sample. This protein migrates as a doublet with a lower mobility of 97 kDa and a higher mobility of 105 kDa (Amersham Biosciences). The immobilized proteins were incubated with primary antibodies against flagella or OmpU followed by incubation with goat anti-rabbit IgG peroxidase (Sigma). All incubations were carried out at room temperature on an orbital shaker unless noted otherwise. The substrate was a chemiluminescent reagent (Amersham Biosciences).

**Two-dimensional Gel Electrophoresis—** Two-dimensional gel electrophoresis was performed according to the method of O’Farrell (45) as follows. IEF was carried out in glass tubes of 2.0-mm inner diameter using 2% pH 4–8 Ampholines (British Drug House, Gallard Schlesinger, Long Island, NY) for 9600 V-h. One microgram of an IEF standard, tropomyosin, was added to each sample. This protein migrates as a doublet with a lower mobility of 97 kDa and a higher mobility of 105 kDa (Amersham Biosciences). The immobilized proteins were incubated with primary antibodies against flagella or OmpU followed by incubation with goat anti-rabbit IgG peroxidase (Sigma). All incubations were carried out at room temperature on an orbital shaker unless noted otherwise. The substrate was a chemiluminescent reagent (Amersham Biosciences).

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RESULTS

The flagella of many bacterial pathogens are capable of activating the production of proinflammatory molecules in epithelial, monocyctic, polymorphonuclear, and dendritic cells (for a review, see Ref. 40). Activation of cytokines such as IL-8, IL-1β, tumor necrosis factor-α, and IL-6 is triggered by the recognition of basolateral TLR5 and by flagellin monomers (36). In this study, we investigated the role of V. cholerae flagella in activating the release of IL-8 in HT-29 human colonic epithelial cells. This cell line has been used in the past to study the ability of bacterial products to induce IL-8 activation (36). It is thought, but has never been demonstrated, that the sheathed polar flagellum of V. cholerae is composed of as many as five distinct flagellins encoded in two chromosomal loci (flaAC and flaDBE) (33). We began this study with the isolation of flagella from V. cholerae 395 classical Ogawa strain (Fig. 1A). This motile strain has been extensively studied and produces CT and TCP (6, 47). Analysis of the purified flagella (Fig. 2C) by SDS-PAGE revealed the presence of two protein bands with apparent molecular masses of 43 and 38 kDa (Fig. 2A). Mass spectrometry analysis of the 43-kDa protein band revealed the presence of several peptides with sequence identity to the three flagellins FlaA, FlaC, and FlaD and to FlgE (flagellar hook protein); however, flagellins FlaB and FlaE were not detected by this technique. The 38-kDa protein band corresponded to OmpU (43). A similar extraction and purification procedure was performed on KKV90, an isogenic flaA mutant that lacks flagella production and motility (33). We confirmed by TEM that this mutant lacks polar flagellum (Fig. 1B) and consequently did not show the 43-kDa protein band in the flagella mock preparation (Fig. 2A). These results were also confirmed by immunoblotting with an antibody against H7 flagella of E. coli (Fig. 2B). Protein sequence comparison between H7 and V. cholerae flagellins showed that there is enough sequence similarity between the two molecules (data not shown), which explains the antigenic cross-reactivity observed by immunoblotting (Fig. 2B). In line with this finding, the H7 antibody, but not the preimmune serum, bound to the purified flagella by immunogold labeling (Fig. 2D).

Bacterial flagella are potent inducers of proinflammatory molecules (for a review, see Ref. 40). Therefore, we investigated whether the V. cholerae flagella were capable of inducing IL-8 release from HT-29 cells. As expected, high levels of IL-8 induction were obtained with heated samples of flagella (representing monomeric flagellin) and not with native flagella (Fig. 3). Boiling is required to dissociate the flagella into flagellin monomers, which are the actual active proinflammatory molecules (36). To confirm this preliminary observation, wild type 395 and KKV90 bacteria and their supernatants were tested for IL-8 activation. It was found that the flaA mutant
was significantly reduced in its capability to trigger IL-8 release; however, it still retained some IL-8-inducing activity (Fig. 4A). In addition, cell-free supernatants of this mutant showed a 20-fold reduction in IL-8-inducing activity (p < 0.0001) (Fig. 4B) from HT-29 cells in comparison with the wild type supernatants (600 pg/ml IL-8). The data suggested that FlmA alone is responsible for most of the stimulation of IL-8 release from HT-29 epithelial cells; however, we sought to identify another molecule(s) in cell-free supernatants of KKV90 accountable for the residual IL-8-inducing activity.

We first determined the best in vitro conditions for secretion of proteins in the wild type and flaA mutant strains. Thus, we used several liquid bacteriological media (LB, Trypticase soy, Brain-heart infusion, Tryptone, and Brucella broth) and grew the bacteria with shaking at 37 °C. No difference in growth rate was observed between these two strains (data not shown). Secreted proteins present in cell-free supernatants were obtained as indicated under “Experimental Procedures” and analyzed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. The results from these experiments indicated that although Brucella broth appeared to be the best media tested, LB broth did not activate secretion of many proteins as well as the other media tested (Table II). Based on these results, we then decided to perform subsequent experiments with bacterial supernatants obtained from bacteria grown until lag, log, and stationary phases of growth in Brucella broth. The reason for this is that the production and secretion of virulence factors in many bacterial pathogens is influenced by the growth phase (4, 48). Concentrations of the secreted proteins were normalized and then analyzed by SDS-PAGE. It is apparent that during midlog growth phase both wild type and flaA mutant strains secreted comparably more proteins in terms of quantity than during lag or stationary phases of growth (Fig. 5A). Notably proteins in the range of 35–43 kDa appear to be increased in concentration during midlog phase in both isolates. In particular, 38- and 43-kDa protein bands were present more abundantly in these preparations. Interestingly, the intensity of bands in the range of 35–43 kDa was more prominent in the mutant than in the wild type.

Immunoblotting of the supernatants showed that the 43-kDa protein reacted with anti-H7 flagella antibody (Fig. 5B), and the intensity of this reaction correlated with the intensity observed in Coomassie Brilliant Blue R-250-stained gels at the different phases of growth (Fig. 5A). The lower 38-kDa protein corresponded to OmpU, a ToxR-regulated outer membrane porin inducible by bile (49). This result indicated that OmpU can be secreted into the extracellular milieu (Fig. 5, A and B).

In an attempt to identify flagellins other than FlmA, we obtained secreted proteins from a collection of V. cholerae 395 isogenic mutants interrupted in more than one flagellin gene (Table I). Given the proximity in size of the five flagellins we electrophoresed the secreted proteins in 10–18% gradient gels at the different phases of growth (Fig. 6A). A doublet band around 43 kDa was seen in most mutants except in the $\Delta$flaAC and the $\Delta$flaBDE strains (Fig. 6A), which

| Media             | Secretion of flagellins |
|-------------------|-------------------------|
| Brucella          | +++                     |
| Tryptone          | +                       |
| Brain-heart infusion | +                     |
| Trypticase soy    | +                       |
| Luria-Bertani     | –                       |

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showed only the larger of the two protein bands seen in the remaining strains. These doublet bands were reactive with anti-flagella antibody, suggesting that they share epitopes with flagellin (Fig. 6B). All of the strains showed a secreted 38-kDa protein band, identified as OmpU, as demonstrated previously by its reactivity with anti-OmpU antibody (Fig. 5B). Differences in a number of proteins outside the range of interest (38–43 kDa) were also found, but our main goal was to identify flagellin proteins. Although helpful, this analysis had its limitation in that we still did not know which of the four remaining flagellin proteins were secreted by the ΔflaA strains, and so we next decided to perform 2-D-GE.

Because the predicted products of the five flagellin genes of V. cholerae possess almost identical molecular masses (40.4 kDa [FlaA], 39.5 kDa [FlaB], 39.9 kDa [FlaC], 39.9 kDa [FlaD], and 41 kDa [FlaE]) (33), we subjected equal concentrations of secreted proteins obtained from 395 and its derivative KKV90 to 2-D-GE. The results illustrated in Fig. 7 clearly show that there are striking differences in the content and number of proteins present in the supernatants of these strains. Obviously we were interested in identifying proteins with molecular

Fig. 5. Secreted proteins profiles of V. cholerae 395 and KKV90 strains. The strains were grown in Brucella broth and measured at different time points. A, in log phase, we noticed the presence of two proteins of molecular masses around 38 kDa (**) and 43 kDa (*). B, the results show that the strain KKV90 still secretes flagellin subunits different from FlaA.

FIG. 6. Secreted protein profiles of V. cholerae strains. A, SDS-PAGE (10–18% gradient) analysis of secreted proteins obtained from V. cholerae strains grown in brucella broth at logarithmic phase. B, immunoblot of secreted proteins from the different strains using anti-H7 flagella serum.

FIG. 7. Proteomics analysis of secreted proteins of V. cholerae 395 and KKV90. Filtered supernatants of 395 (A) and KKV90 (B) grown in Brucella broth were analyzed by 2-D-GE. Proteins in the range of 30–65 kDa were subjected to mass spectrometry for identification. Both strains secreted OmpU (spot 1), an outer membrane porin. Also we found flagellin FlaC (spot 2), FlaD (spot 3), flagellar hook protein FlgE (spot 4), chaperonin Profla (spot 5), FlaA (spot 6), TolC (spot 7), and OmpV (spot 8).
masses between 38 and 43 kDa, but we were also curious about the 66-kDa protein because flagellins of the enterobacteria are proteins of similar size.

The circled spots in Fig. 7 indicate proteins that were subjected to mass spectrometry for identification, and the results are summarized in Table III. Spot 1, which is present with equal intensity in both strains, corresponds to OmpU of 38 kDa (National Institute of Biotechnology Information (NCBI) accession number GI:1658032) (43). Spot 2 is FlaC, identified as a 39.9-kDa flagellin protein (NCBI accession number GI:9656742), which appeared to be secreted more abundantly in the flaA mutant than in the wild type. Spot 3 corresponds to FlaD (NCBI accession number GI:9656695), another prominent flagellin protein of 39.77 kDa found to be secreted by KKV90. Spot 4 is a 46.86-kDa protein identified as FlgE (NCBI accession number GI:9656753), a flagellar hook protein that also appears to be more prominent in supernatants of the mutant flaA strain. Spot 5 matches the sequence of a 60-kDa chaperonin Profla protein (NCBI accession number GI:9657257). Spot 6 was identified as FlaA (NCBI accession number GI:9656743), the major flagellin (39.8 kDa) component of the V. cholerae 395 flagellum, and was not found in the flaA mutant (Fig. 2). Spot 7 was identified as outer membrane protein TolC (47.7 kDa) (NCBI accession number GI:9657012), which contains a β-barrel domain and is involved in transport of many molecules across the cell envelope (50), and spot 8 corresponds to outer membrane protein OmpV (24.65 kDa) (NCBI accession number GI:9655807). Other proteins outside the range studied were also present in the supernatant, but they were not analyzed.

Because proteins obtained from KKV90 were more abundant than in the parent strain, we chose to perform immunoblotting analysis of secreted proteins obtained from KKV90. A 2-D-GE identical to that shown in Fig. 7B containing KKV90 secreted proteins was blotted and incubated with anti-flagella and anti-OmpU antibodies. We found that the two flagellins FlaC (spot 2) and FlaD (spot 3) were recognized by anti-flagella antibody (Fig. 8A). The protein in spot 1, corresponding to OmpU, was recognized by the anti-OmpU antibody as expected, and it also showed cross-reactivity with anti-flagella antibody (Fig. 8B).

The proteomics approach allowed us to identify two flagellin proteins, FlaC and FlaD, that appear to be secreted into the extracellular milieu. Thus, we decided to construct double isogenic mutants flaAC and flaAD as a genetic approach to confirm the role of the FlaC and FlaD flagellins as inducers of proinflammatory activity, namely activation of IL-8 release from HT-29 cells. Also available from a previous study (33) were double and triple mutants in different flagellin genes (Table I). Bacterial cultures and cell-free supernatants containing secreted proteins of all of the strains listed in Table I were studied for their ability to activate IL-8 secretion from HT-29 cells.

When bacterial cultures were analyzed, the amount of IL-8 detected in supernatants of HT-29 cells infected with the flaA mutant was significantly reduced 3-fold compared with the wild type strain (p < 0.0001) (Fig. 4A). Single flaC and flaD mutants did not show any reduction in IL-8 release suggesting that FlaA is the most dominant antigen in inducing activation of IL-8 secretion. Significant reduction in IL-8 secretion (p < 0.0001) was observed in response to the double mutants flaAD and flaAC compared with the flaA mutant.

Next we analyzed the supernatants of HT-29 cells incubated with the secreted proteins of the various flagellin mutants. We found that the supernatants of flaAC and flaAD double mutants induced almost no IL-8 release from HT-29 cells, firmly establishing that, in addition to FlaA, FlaC and FlaD are also potent inducers of inflammation (Fig. 4B). Other isogenic strains carrying double, triple, and quadruple mutations in some of the five flagellin genes, namely flaBDE,
flaCDE, and flaBCDE, were also reduced significantly \((p < 0.0001)\) in triggering activation of IL-8 secretion from HT-29 cells. However, the most dramatic effect observed was that of the double flaAC and flaAD mutants. OmpU did not appear to possess a proinflammatory activity because no significant difference \((p < 0.7001)\) was observed between the flaA and the ompUflaA mutants (data not shown).

**DISCUSSION**

*V. cholerae* produces, in addition to the potent CT, several cytotoxic factors including HAP, Hly, and RTX (9–11). Since the discovery of these additional putative virulence factors, several studies using isogenic mutants deficient in production of these cytotoxins have suggested that they may cause tissue damage resulting from proinflammatory responses. HAP affects paracellular barrier function in epithelial cells by degrading tight junctions and hydrolyzing mucin, which has been hypothesized to enhance detachment of *V. cholerae* from epithelial cells (10). Hly causes necrosis of intestinal epithelial cells and vacuolation of cells (11, 51). Lastly RTX induces cell rounding and increased permeability through paracellular tight junctions due to cross-linking of actin monomers leading to depolymerization of actin stress fibers (9).

Given the global impact of cholera, several oral live attenuated vaccines have been constructed aimed at protecting people living in cholera-endemic areas and travelers to these areas. These CT-negative vaccine strains contain single or multiple mutations in *hap*, *rtx*, and *hly* cytotoxin genes and adherence factor *tcpA*, *cep*, and *acf* genes, and although protective, there appears to be some residual non-desirable side effects associated with these vaccines (1, 12, 15–20). There are indications that these CT-minus strains may trigger an inflammatory response manifested by infiltration of polymorphonuclear neutrophils, rise in lactoferrin levels, and enterocolitis (26, 27). Cholera is a secretory non-inflammatory disease, and it has been suggested that this is because CT has anti-inflammatory properties (27). Nonetheless only RTX mutants have been demonstrated in a murine pulmonary cholera model to show less severe pathology and decreased serum levels of proinflammatory IL-6 and murine macrophage inflammatory protein-2 suggesting that RTX participates in the severity of acute inflammatory responses (12). However, a recent study suggested that neither *hap* nor *rtx* deletion mutants showed any differences in induction of IL-8 release from T84 colonic cells compared with the wild type strain (28).

Previous studies in human volunteers that were fed with derivatives of a Peruvian strain of *V. cholerae* O1, biotype El Tor, namely Peru-3 (a flagellated vaccine strain) and Peru-15 (a non-flagellated vaccine strain), indicated that although both were significantly protective only Peru-15 was non-reactogenic, indicating that flagella may be responsible for some of the adverse reactions seen with Peru-3 and other vaccine strains (15, 20). In sum, the proinflammatory component associated with the reactogenicity of CT-negative *V. cholerae* vaccine strains remains elusive.

Flagellum-driven motility is required for full virulence of *V. cholerae* (for a review, see Ref. 40) (52–54). Five distinct flagellin genes (*flaAC* and *flaBCDE*) are transcribed within motile strains of *V. cholerae* (33). The flagella of *Vibrio paraheamolyticus* and *Vibrio anguillarum* are composed of multiple flagelin subunits similar in molecular mass (55, 56). Mutations in several flagellin genes of *V. anguillarum* led to significant defects in virulence in animal models without abolishing it entirely, indicating that multiple flagellins may play roles in virulence (56). By analogy to *V. paraheamolyticus* and *V. anguillarum*, it is thought that the five flagellins of *V. cholerae* are also assembled into the flagellum filament, but this has never been formally proven. Furthermore only a mutation in *flaA* abolishes motility, suggesting that the remaining four flagellin proteins are not essential for flagellum structure and function at least under laboratory growth conditions (33). Here we found that purified *V. cholerae* flagella contain FlaA, FlaC, and FlaD as determined by the mass spectrometry analysis; however, the other two flagellins, FlaB and FlaE were not detected.

We undertook genetic and biochemical approaches to identify the secreted product(s) of *V. cholerae* responsible for activating proinflammatory cytokines and perhaps of the reactogenicity inherent to CT-minus strains. Although flagellins of many bacterial pathogens have been shown to be potent activators of proinflammatory cytokines via activation of cellular basolateral TLR5 (36), this activity has not been demonstrated for *V. cholerae* flagelin. We found that the purified flagellum induced the release of significant levels of IL-8 from HT-29 cells. This result is relevant given that reactogenic *V. cholerae* O1 vaccine strains are flagellated, whereas non旗ellated strain Peru-15 is protective and non-reactogenic (15, 20). Supernatants of a non-flagellated ΔflaA strain showed a 20-fold reduction in IL-8 release in comparison with the supernatant from the wild type strain, demonstrating a major proinflammatory role for FlaA. However, measurable residual IL-8 stimulatory activity existed in the supernatant of the ΔflaA strain.

A comparative proteomics analysis using 2-D-GE and mass spectrometry of secreted proteins contained in cell-free supernatants of the wild type and flaA mutant strains identified two additional flagellin proteins, FlaC and FlaD, that appeared to be secreted more abundantly in KKV90 than in 395 perhaps to compensate for the loss of FlaA in the mutant. We sought to determine whether the residual IL-8-inducing activity in the supernatant of KKV90 was due to these specific flagellins. Analysis of inflammatory responses to *V. cholerae* strains with single and multiple mutations in the five flagellin genes led to the finding that FlaC and FlaD are also important proinflammatory molecules as induction of IL-8 release from HT-29 cells by supernatants of the double flaAC and flaAD mutants was practically abolished. There may be additional components not identified here that also contribute to inflammatory
response in vivo; for example, FlaB or FlaE could not be detected in the supernatants studied; however, it is possible that they might be secreted in the gut environment and have IL-8 induction capability. All of the flagellin genes are expressed in vitro, and flaBCDE have been demonstrated to be expressed in a flaA mutant (33). It is clear that among the V. cholerae flagellins found here FlaA showed the highest proinflammatory activity.

Another interesting finding in this study is that OmpU, an outer membrane porin regulated by the virulence regulator ToxR (57), was secreted into the supernatant. Because the flagellum of V. cholerae is sheathed and thought to be composed of one outer membrane protein (35), we reasoned that perhaps OmpU might correspond to this protein. However, we could not find a physical or structural association of OmpU with the flagellum as determined by immunogold labeling studies. Furthermore we could not find an association of OmpU and activation of IL-8 secretion; thus it remains to be determined what role secreted OmpU plays in the interaction of V. cholerae with host epithelial or immune cells given that it normally functions as an integral membrane porin with relative cationic specificity (49). In all, our results may help explain the reactogenicity associated with V. cholerae strains lacking CT.

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