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Structural and Functional Importance of Outer Membrane Proteins in *Vibrio cholerae* Flagellum

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***Vibrio cholerae* has a sheath-covered monotrichous flagellum that is known to contribute to virulence. Although the structural organization of the *V. cholerae* flagellum has been extensively studied, the involvement of outer membrane proteins as integral components in the flagellum still remains elusive. Here we show that flagella produced by *V. cholerae* O1 El Tor strain C6706 were two times thicker than those from two other Gram-negative bacteria. A C6706 mutant strain (SSY11) devoid of two outer membrane proteins (OMPs), OmpU and OmpT, produced thinner flagella. SSY11 showed significant defects in the flagella-mediated motility as compared to its parental strain. Moreover, increased shedding of the flagella-associated proteins was observed in the culture supernatant of SSY11. This finding was also supported by the observation that culture supernatants of the SSY11 strain induced the production of a significantly higher level of IL-8 in human colon carcinoma HT29 and alveolar epithelial A549 cells than those of the wild-type C6706 strain. These results further suggest a definite role of these two OMPs in providing the structural integrity of the *V. cholerae* flagellum as part of the surrounding sheath.**

Keywords: *Vibrio cholera*, flagella, outer membrane proteins

Introduction

Vibrio cholerae is a highly motile, Gram-negative bacterium that causes the severe diarrheal disease, cholera. Similar to many other pathogenic bacteria, flagella-mediated motility plays an important role in *V. cholerae* virulence (Richardson, 1991; Gardel and Mekalanos, 1996; Yoon and Mekalanos, 2008). Moreover, spontaneous non-motile *V. cholerae* mutants, which elicit reduced disease symptoms, have been successfully tested as oral cholera vaccines further supporting the *in vivo* role of the flagellum in human infection (Coster *et al.*, 1995; Kenner *et al.*, 1995; Qadri *et al.*, 2007). The single polar flagellum of *V. cholerae* is mainly composed of five flagellin proteins, FlaA, B, C, D, and E (Klose and Mekalanos, 1998; Yoon and Mekalanos, 2008). The flagellin proteins were found to be the major reactogenic agent of the live attenuated *V. cholerae* vaccine (Rui *et al.*, 2010). A hallmark of the *V. cholerae* flagellum is the presence of a sheath around the flagella protofilament (Follett and Gordon, 1963; Yoon and Mekalanos, 2008). While the sheath has been postulated to be a continuous extension of the outer membrane (OM) (Hranitzky *et al.*, 1980; Richardson and Parker, 1985; Fuerst and Perry, 1988), molecular constituents of the sheath and its functional role in motility have not clearly been defined. Recently, it was reported that *V. cholerae* flagella induced a significantly lower toll-like receptor (TLR) 5-mediated host inflammatory response, when compared with sheathless flagella derived from *Salmonella enterica* serovar Typhimurium (Yoon and Mekalanos, 2008). The reduced potency of the *V. cholerae* flagella was attributed to repressed flagellin shedding, most likely due to the presence of the sheath.

V. cholerae expresses an array of outer membrane proteins (OMPs) with a range of subunit molecular masses (Sengupta *et al.*, 1992). Among these OMPs, OmpU, and OmpT have been studied extensively in terms of virulence, adherence, and colonization in the host (Singh *et al.*, 1994; Sperandio *et al.*, 1995; Provenzano and Klose, 2000). In *V. cholerae*, the transcriptional activator ToxR, a transmembrane protein that mediates the coordinate expression of virulence factors (Miller *et al.*, 1987), also differentially regulates the transcription of *ompU* and *ompT* (Miller and Mekalanos, 1988; Chakrabarti *et al.*, 1996; Champion *et al.*, 1997; Crawford *et al.*, 1998; Li *et al.*, 2000). It has been shown that OmpU and OmpT have porin properties (Chakrabarti *et al.*, 1996; Wibbenmeyer *et al.*, 2002; Simonet *et al.*, 2003) and are differentially affected by bile *in vitro* and *in vivo* (Provenzano and Klose, 2000; Wibbenmeyer *et al.*, 2002; Duret and Delcour, 2006). Despite all of these studies, there is a lack of information regarding the structural importance of these OMPs in the *V. cholerae* flagellum as a part of the sheath structure, except for some discrete reports regarding the involvement of OMPs with flagella core proteins (Hranitzky *et al.*, 1980; Richardson and Parker, 1985).

In this study we demonstrated that OmpU and OmpT are associated with the structural organization of the *V. cholerae* flagellum as essential components of the flagellar sheath. A mutant strain devoid of both OMPs produced thinner flagella and exhibited a greater tendency to lose flagellar structural integrity. This study thus provides further evidence that the *V. cholerae* flagellar sheath might be an inherent extension of the OM surrounding the flagellum.

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Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotype or description	Reference or source
Strains		
V. cholerae		
C6706	7th pandemic O1 serotype, El Tor biotype	Lab collection
C6706, Δ*hlyA*	C6706, *hlyA* deletion mutant	This study
SSY10	C6706, *ompU*::Tn	Yoon and Mekalanos (2008)
SSY10, Δ*hlyA*	SSY10, *hlyA* deletion mutant	This study
SSY11	SSY10, Δ*ompT*	This study
SSY11, Δ*hlyA*	SSY11, *hlyA* deletion mutant	This study
P. aeruginosa		
PAO1	Wild-type lab strain	Lab collection
S. enterica		
LT2	Wild-type lab strain	Lab collection
E. coli		
DH5α	*supE44* Δ*lacU169* (φ80d*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab collection
SM10λ*pir*	Km^r*thi-1 thr leu tonA 16acy supE recA*::RP4-2-Tc::Mu *pir*$^+$	Lab collection
Plasmids		
pWM91	*sacB* suicide vector, Ap^r used for *ompT* deletion	Lab collection
pCVD442-Cm	*sacB* suicide vector with Cm^r used for *hlyA* deletion	This study
pIndigoBAC-5	Source of Cm resistant gene	Lab collection

Materials and Methods

Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in LB medium (10 g tryptone, 5 g NaCl, and 5 g yeast extract per L) with appropriate antibiotics at the following concentrations: for *V. cholerae* streptomycin (200 μg/ml), kanamycin (100 μg/ml), and chloramphenicol (6 μg/ml), for *E. coli* kanamycin (50 μg/ml), unless otherwise mentioned.

Construction of mutant strains

A sequence-verified *ompU* deficient mutant of the C6706 (SSY10) was obtained from a non-redundant transposon insertion mutant library (Cameron *et al.*, 2008). An in-frame *ompT* deletion mutation was generated using the *ompU* deficient SSY10 strain to construct an *ΔompUΔompT* double mutant strain (SSY11) following a gene replacement procedure involving sucrose counter selection (Schweizer and Hoang, 1995). Introduction of *hlyA* gene mutation was achieved by allelic exchange as previously described (Philippe *et al.*, 2004). Seven hundred base pair flanking sequences at both ends of the *hlyA* locus were PCR-amplified with primers VCA0219 L(F)-TCTA**GTCGAC***GGCGGGTTCATTTTG ATTGC (*Sal*I), VCA0219 L(R)-GAATT**GAGCTC***GTGA ATATCGCGATTGCGCA (*Sac*I), VCA0219 R(F)-CACTA **GAGCTC***TCGTTTGTATGTGCGAGCGG (*Sac*I) and VCA 0219 R(R)-GAATT**CCCGGG***CGCATCCGAGGAATACG TGAG (*Sma*I) harboring specific restriction enzyme sites. The *hlyA* upstream and downstream sequences were restricted with *Sal*I and *Sac*I and *Sac*I and *Sma*I, respectively. The pCVD442-Cm suicide vector for gene replacement, carrying a chloramphenicol resistance marker, was cleaved with *Sal*I and *Sma*I, and ligated with the respective PCR products. The resultant suicide vectors carrying the *hlyA* gene deletion were electroporated into *E. coli* SM10λ*pir* for subsequent conjugation into *V. cholerae* strains. Transconjugants were selected on LB agar plates containing both 200 μg/ml streptomycin and 8 μg/ml chloramphenicol and the second crossover of allelic exchange was induced by 10% sucrose. Deletion of the *hlyA* gene locus was confirmed by PCRusing the primers *hlyA*destruct Idn-(F) ACACCCAGCGGCTTCCCTAG and *hlyA* destruct Idn-(R) CCGCTTGCTGCAAAAGCGTA.

Motility assay

LB plates containing 0.3% agar (Difco, USA) were spot inoculated with overnight-grown cultures of wild-type and OMP mutant strains of *V. cholerae*. The plates were incubated at 37°C for 16–18 h and measured for swimming diameter (Gupta and Chowdhury, 1997).

Preparation of purified flagella

Flagella purification was accomplished following the procedure described previously (Bari *et al.*, 2011). Purity of purified flagella was analyzed by 12% SDS-PAGE.

TCA precipitation of culture supernatants

Total protein content was precipitated from filter-sterilized culture supernatants of overnight grown *V. cholerae* cultures using trichloroacetic acid (TCA). Briefly, 1 volume of 100% (W/V) TCA was added to 4 volumes of each culture supernatant (protein sample) and incubated for 10 min at 4°C. The mixture was then centrifuged at 14,000 rpm for 5 min. Supernatants were removed leaving the protein pellet intact. The pellet was then washed with 200 μl ice-cold acetone and was again centrifuged at 14,000 rpm for 5 min. After repeating the washing step twice, the pellet was allowed to dry in a 95°C heat block to drive off the acetone. The completely dried pellet was mixed with 5× sample buffer and boiled for 5 min before loading onto an 8% SDS-PAGE gel.

Western blot analysis and mass spectrometry

Equal amounts of total protein from whole cell extracts and flagellar preparations were separated by 12% SDS-PAGE gels and transferred to PVDF membranes (Amersham, Hybond-ECL, GE Healthcare). Membranes were probed with rabbit polyclonal anti-OmpU antiserum (Sperandio *et al.*, 1995) at a 1:500 dilution at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (Santa Cruz Biotechnology, USA). The immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL) reagent WESTSAVE Up (Ab Frontier, USA). Protein bands were visualized by Coomassie Brilliant Blue and proteins of interest were further identified by mass spectrometry (Yoon and Mekalanos, 2008).

Electron microscopy

Bacterial cultures grown to mid-log phase were harvested (500 µl) and resuspended in 500 µl PBS. The bacterial suspension (10 µl) was applied to a carbon-coated, copper mesh grid prepared with plasma discharge using an Edwards E306A vacuum coating system (Edwards High Vacuum, UK) immediately beforehand. The suspension was allowed to make contact for 1 min and then excess fluid was drained away by soaking on a blotting membrane. Ten microliter of 1% uranyl acetate (pH 6.5) was added for 1–2 min and excess dye was removed in the same manner. Prepared samples were viewed using a Philips CM100 transmission electron microscope (Philips/FEI Corporation, Holland) operating at 100 kV and images were captured digitally using a Mega-View 3 camera (Olympus Soft Imaging Solutions GmbH, Germany). The flagellar diameter was measured using iTEM acquisition and analysis software (Olympus Soft Imaging Solutions GmbH).

Cell culture, reagents, and stimulation

Intestinal epithelial HT29 and alveolar epithelial A549 cell lines (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM), respectively, supplemented with 10% FBS (WelGENE, Korea), 2.5 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C under 5% CO_2 in a water-jacketed CO_2 incubator (Forma Scientific, USA). For stimulation with bacterial cell-free culture supernatants, 2×10^4 hostcells were seeded in each well of 96-well plates (SPL Life Sciences, Korea) and cultured overnight. The next day, the culture medium was replaced with serum-free medium before treatment. Prepared culture supernatants (10 µl) from bacterial cultures were added to cultured host cells and incubated for 6 h at 37°C under 5% CO_2. Samples for IL-8 ELISA assays were collected and cell viability was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) according to the manufacturer's instructions.

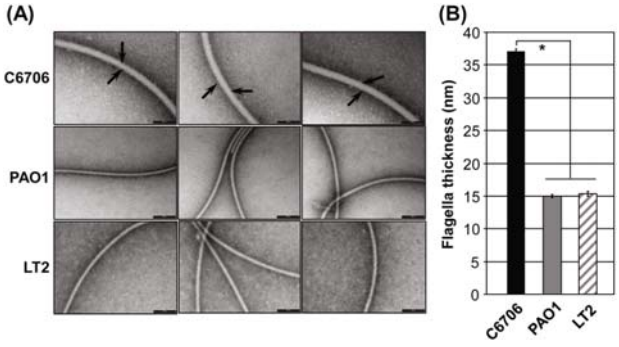

Fig. 1. Image analysis of flagella derived from three different, Gram-negative, bacterial species. (A) Representative TEM images of flagella of *V. cholerae* O1 strain C6706, *P. aeruginosa* PAO1 and *S. enterica* serovar Typhimurium LT2. A scale bar (black line) of 100 nm is shown in each image. Black arrows in the topmost row indicate two separate layers of the *V. cholerae* flagella. (B) Quantitative measurement of flagellar thickness. Flagellar diameters were calculated as described in the 'Materials and Methods'. Sixteen, 19 and 22 images were saved for diameter calculation for C6706, PAO1, and LT2 strains, respectively (*p<0.005).

IL-8 assay

HT29 and A549 cell culture supernatants were collected after treatment and stored at -80°C. IL-8 production was determined using the HU IL-8 CYTOSET ELISA kit (Invitrogen Corp., USA) according to the manufacturer's instructions. Titers are expressed as pg/ml.

Statistical analyses

Data are expressed as the mean±SD. An unpaired Student's t-test was used to analyze the data. A p-value of <0.05 was considered statistically significant. All the experiments were repeated for reproducibility.

Results

V. cholera flagella are thicker than flagella from other bacterial species

To determine unique physical features that distinguish the *V. cholerae* flagellum from those derived from two other Gram-negative pathogens, *Pseudomonas aeruginosa* strain PAO1 and *Salmonella enterica* serovar Typhimurium strain LT2 (Table 1), we visualized bacterial flagella using transmission electron microscopy (TEM). As shown in Fig. 1A, flagella attached to C6706 cells were more than two-times thicker (>36 nm) than those of prototype PAO1 and LT2 strains, while the thicknesses of flagella produced from PAO1 and LT2 were indistinguishable (Fig. 1B). Moreover, two distinct layers were clearly observed for C6706 flagella (arrow, Fig. 1A). Two other *V. cholerae* strains, O395 (O1/classical) and N16961 (O1/El Tor) produced flagella that are similar to those of C6706 (data not shown). This result suggests that *V. cholerae* flagella are unusually thick compared to other non-sheathed flagella of Gram-negative pathogens.

OMP fractions are co-extracted with flagella during purification

Next, we analyzed purified flagella from *V. cholerae* by SDS-PAGE to identify any non-flagellin protein constituents. Purified flagella derived from PAO1 or LT2 were mainly composed of flagellin monomers as no other major protein bands were detected (Fig. 2A). In contrast, we identified

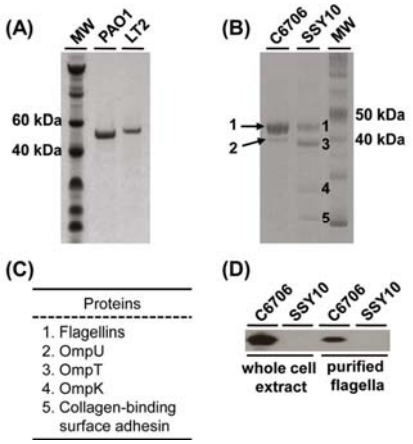

Fig. 2. Protein analysis of purified flagella. (A) Purified flagellar samples extracted from LB-grown PAO1 and LT2 strains (1 μg) were loaded onto a 12% SDS-PAGE gel. MW, molecular weight marker in kDa. (B) Purified flagellar samples extracted from *V. cholerae* C6706 and SSY10 strains were analyzed by 12% SDS-PAGE. (C) Identification of proteins that were detected in the SDS-PAGE gel (panel B). (D) Western blot analysis of OmpU in the whole cell extracts and purified flagella of C6706 or SSY10.

OmpU in the purified flagella of C6706 (Fig. 2B, band 2) as determined by mass spectrometric analysis. Next, we examined the effect of an *ompU* deletion on flagellar composition. Interestingly, we detected a significantly enhanced level of OmpT in the flagella purified from the OmpU deficient C6706 mutant, SSY10 (Fig. 2B, SSY10, band 3). In addition, two additional OMPs, OmpK (band 4), and VC1894 (predicted collagen-binding surface adhesion, band 5), were identified in the purified flagella of SSY10 (Figs. 2B and 2C) by mass spectrometry. To ensure the co-extraction of OmpU with flagella, we performed western blot hybridization using anti-OmpU antiserum. The presence of OmpU was detected at a substantial level in both whole cell extracts and in purified flagella (Fig. 2D); however, we failed to isolate pure flagella from SSY11, even after extensive attempts using numerous experimental permutations (data not shown). Such an observation may suggest that the presence of OMPs is required for the purification of pure flagella in *V. cholerae*.

Elevated level of flagellin proteins was detected in the culture supernatants of SSY11

We then sought to compare the protein content present in the culture supernatants of OMP mutant strains with that of the wild-type strain. After performing SDS-PAGE with filter-sterilized and TCA-precipitated culture supernatants, a thick flagellin protein band (Fig. 3A, no. 1) was detected in all tested culture supernatants, with a higher amount in the sample derived from SSY11. The *ompU* defective mutant strain SSY10 also produced the same sharp band corresponding to the molecular weight of the flagellin proteins but the band appeared less intense than the band from SSY11, when scored visibly after Coomassie Brilliant Blue staining (Fig. 3A). Another interesting finding was the detection of flagella hook-associated protein VC2191 (Fig. 3, no. 2) in the TCA-precipitated culture supernatant of SSY11. This protein band was not found in the SSY10 or wild-type C6706

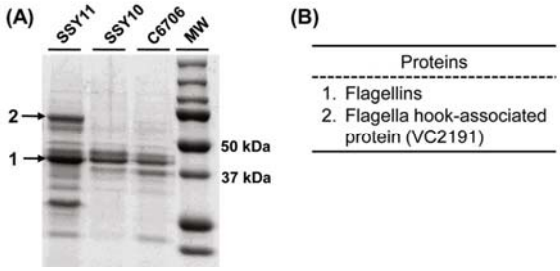

Fig. 3. Protein profile of culture supernatants from wild-type and OMP mutant strains. (A) Culture supernatants of overnight-grown C6706 and the OMP mutants were concentrated by TCA precipitation and analyzed by SDS-PAGE. MW, molecular weight marker. (B) List of proteins identified by mass spectrometry.

strains. This result strongly suggests that flagellin shedding is increased to a greater extent in the mutant strain lacking both OmpT and OmpU.

OMP deletion mutant strains produced thinner flagella and were less motile

To further elucidate the structural role of OMPs in the *V. cholerae* flagellum, flagella attached to C6706, SSY10 or SSY11 cells were visualized by TEM. As shown in Fig. 4A, flagella of SSY11 looked thinner than those derived from the wild-type C6706 or its isogenic OmpU mutant strain, SSY10. Analysis of software-aided distance measurements with digitized flagella images indicated that flagella derived from C6706, SSY10 and SSY11 were 36.45 (±0.286, n=15), 34.07 (±0.288, n=23), and 28.71 (±0.238, n=36) nm in diameter, respectively (Fig. 4B). Flagella of SSY10 were similar to those of C6706 in cross-sectional diameter, most likely due to the upregulation of OmpT in their flagella (Fig. 2B). These results strongly suggest that OMPs, especially OmpU and OmpT (the latter, when OmpU is not present), are likely major structural components of the *V. cholerae* sheathed flagellum. Next, we tested if the functional motility of SSY11

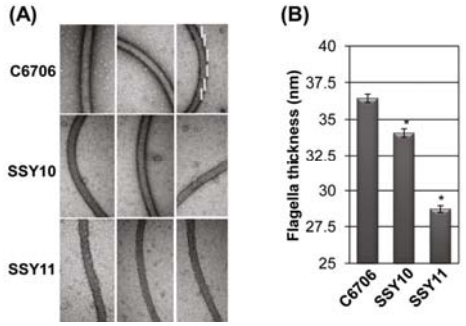

Fig. 4. Image analysis of flagella derived from three different *V. cholerae* strains. (A) Representative TEM images of flagella of the *V. cholerae* O1 C6706, SSY10, and SSY11 strains. Experimental conditions were identical to those described for Fig. 1A. (B) Quantitative measurement of flagellar thickness. Flagellar diameters were calculated as described in the 'Materials and Methods'. Sixteen, 23 and 36 images were saved for diameter calculation for C6706, SSY10, and SY11 strains, respectively (*p<0.001 vs. flagellar diameter of C6706).

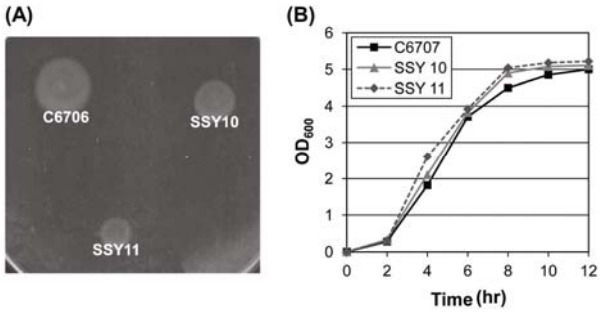

Fig. 5. Reduced flagella-mediated motility of the SSY11 strain. (A) C6706, SSY10, and SSY11 strains were spot-inoculated in 0.3% LB agar and incubated for 6 h at 37°C. (B) Representative growth curves of tested strains. Stains were grown in LB broth for 12 h.

was affected by the lack of two major OMPs in the flagellum. As shown in Fig. 5A, SSY11 showed a significant defect in motility compared to its parental strain, suggesting that the proper incorporation of OMPs into the sheathed *V. cholerae* flagellum is necessary for their function. SSY10 exhibited an intermediate motility level between WT and SSY11. Similar specific growth rates were observed for SSY11, SSY10, and C6706 indicating that the reduced motility was not due to affected growth (Fig. 5B).

Culture supernatants of SSY11 induced production of higher levels of IL-8 in epithelial cells

Cultured epithelial cells respond to the bacterial flagella via the TLR5-mediated innate immune system (Harrison *et al.*, 2008). Since the OMP mutant strains were shown to release more flagellin proteins to the culture media during growth, we compared the level of IL-8 produced by HT29 and A549 epithelial cells in response to the treatment with culture supernatants containing varying degrees of flagellar content. As shown in Fig. 6A, the culture supernatant from the *ΔompUΔompT* double mutant strain, SSY11, induced significantly higher levels of IL-8 (~408 pg/ml) compared to the culture supernatant of wild-type C6706 strain (~238

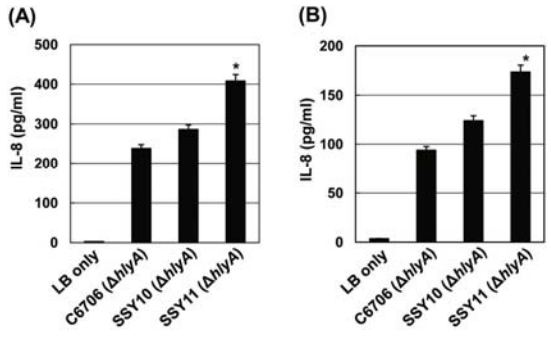

Fig. 6. Higher production of the proinflammatory cytokine, IL-8 by OMP mutant strains. Culture supernatants of *hlyA*-negative C6706, SSY10 and SSY11 strains were used to treat (A) HT29 and (B) A549 cells. ELISA assays were performed to determine the level of IL-8 and values of means±SDs are displayed in each bar (n=6). *$p<0.05$ for comparisons between the wild-type and double mutant type culture supernatants.

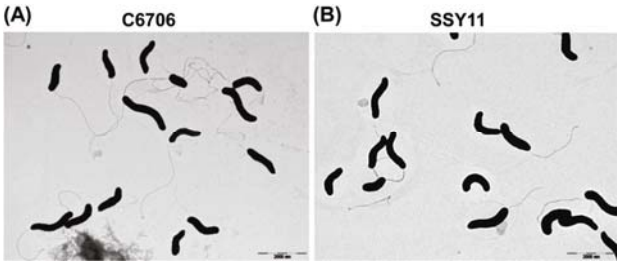

Fig. 7. Increased abundance of non-flagellated bacterial cells in an SSY11 cell population. Representative TEM images of C6706 cells (A) and SSY11 cells (B). Images were acquired and processed at a magnification of 10,000×.

pg/ml) in HT29 intestinal epithelial cells. Culture supernatants derived from the *ΔompU* mutant strain SSY10 also induced higher levels of IL-8, although the difference was not statistically significant compared to the wild-type supernatant. These data further confirm the presence of an elevated level of flagella in the SSY11 culture supernatants. It should be noted that an in-frame deletion of the *hlyA* gene encoding the hemolysin toxin was generated in all tested strains (C6706, SSY10, and SSY11) to maintain epithelial cell viability, as the *V. cholerae* hemolysin, secreted to the culture media, is cytotoxic to the cultured epithelial cells (Coelho *et al.*, 2000). To corroborate our findings with HT29 cells, we also treated A549 cells, another widely-used epithelial cell line, with the identical set of culture supernatants. As expected, the IL-8 production profile was quite similar in the A549 cells (Fig. 6B).

The SSY11 strain contains more aflagellated cells in the population

As we found that the SSY11 mutant strain released more flagellin proteins to the environment, we compared the relative abundance of bacterial cells possessing an intact flagellum in given samples of the WT and SSY11 cell populations. As anticipated, a significant portion of SSY11 cells were found to be devoid of flagella (Fig. 7B). In contrast, almost all the wild-type C6706 bacterial cells had a single, attached polar flagellum (Fig. 7A). This observation further corroborates the role of two major OMPs in the maintenance of structural integrity of the *V. cholerae* flagellum.

Discussion

Flagellum-mediated motility has long been considered as a virulence factor for *V. cholerae*, because the loss of motility reduces its infectivity (Gardel and Mekalanos, 1996). To understand the pathological effects exerted by the flagella, it is important to know the structural details of this organelle. Although the molecular structure of flagella has been a topic of intense research, relatively fewer studies have been performed with regard to the importance of the flagellar sheath and the involvement of OMPs in the sheath structure. Recently, two OMPs, FlgO and FlgP have been identified to play a role in *V. cholerae* motility suggesting a potential in-

volvement of OMPs in motility (Martinez *et al.*, 2009). The purpose of this study was to show that the *V. cholerae* flagellum also requires two other extensively studied OMPs, OmpU and OmpT for its structural and functional integrity.

V. cholerae flagellum appeared much thicker probably due to the presence of the surrounding sheath. Flagella derived from *P. aeruginosa* or *S. enterica* serovar Typhimurium were considerably thinner and characteristic fine layers observed in the images of *V. cholerae* flagella were not detected (Fig. 1). This interesting observation demonstrated that the *V. cholerae* sheathed flagellum has a unique structural organization and deserved further investigation.

Analysis of the purified flagella from *V. cholerae* and two other Gram-negative species provided an initial insight into the structural details, because an OMP (i.e., OmpU) was specifically detected in the *V. cholerae* flagella. Consistent with this result, OmpU was also detected in the purified flagella of *V. cholerae* classical biotype strain O395 (Xicohtencatl-Cortes *et al.*, 2006). Co-purification of OmpU was further evidenced by western blot analysis. On the other hand, flagellin core proteins were also detected in the OM fraction of *V. cholerae* (Richardson and Parker, 1985). Collectively, these results suggest that these two structural components (i.e., the flagellum and OM) are closely associated, such that these two are co-purified. Interestingly, there was an up-regulation of OmpT and other OMPs, when the *ompU* gene was disrupted (Fig. 2). These results suggest that *V. cholerae* might have developed a regulatory mechanism to ensure the incorporation of other OMPs, such as OmpT into the flagellar structure, when OmpU is not available. Failure to extract pure flagella from the mutant strain lacking both OmpU and OmpT provided an additional clue that OM fractions are essentially required to maintain the structural integrity of the *V. cholerae* flagellum. Therefore, OMPs appear to be obligate components of the *V. cholerae* flagellum.

Protein content analysis of the culture supernatants harvested from the wild-type C6706, SSY10 and SSY11 strains revealed that flagellin proteins are released into the culture media, with the SSY11 strain releasing the highest amount (Fig. 3). It is well known that a flagellin protein is a potential immune modulator and is able to induce IL-8 by TLR5-mediated innate immune responses (Harrison *et al.*, 2008). Two different epithelial cells produced increased levels of IL-8, a proinflammatory cytokine, in response to the treatment with the culture supernatant of the SSY11 strain (Fig. 6). Importantly, we also identified a flagellar hook-associated protein (FlgK) in the culture supernatant of SSY11. Considering the fact that flagellar hook-associated proteins are located at the basal body of the flagellar structure, the shedding of this particular protein may suggest that a significant structural anomaly exists in the flagella of the SSY11 mutant.

Our observation that the SSY11 strain possesses a reduced swimming motility could be explained in two different manners (i) the physical structure of the flagellum may be adversely affected due to the lack of two OMPs, or (ii) the flagellar rotation may not operate functionally in the mutant. The first proposition was likely supported by our TEM data, which clearly show that the SSY11 strain produced much thinner flagella, compared to its parental C6706 strain (Fig. 4). Thus, the reduced motility might be caused by the thinner or faulty flagellum structure. Whether the deletion of these OMPs exerts any impact on the flagellar rotation is still unknown and requires future investigation.

Additional experimentation including the measurement of flagellar length may provide an additional insight into the importance of OMPs in the flagellar structure. Unfortunately, we were unable to measure the flagellar length from the digitized TEM images of flagella due to the folding and spiral array of the flagellar filaments. *V. cholerae* flagella consist of five different flagellin monomers (Yoon and Mekalanos, 2008). Given that flagella of *P. aeruginosa* or *S. enterica* serovar Typhimurium are composed of a single flagellin protein, we also hypothesized that the flagellar thickness may be affected by the number of flagellin monomers incorporated into the flagellar filaments. A non-flagellated strain CVD120 (Harrison *et al.*, 2008) was complemented with a *flaA* gene to produce flagella composed of a single monomeric flagellin and then the thickness was compared with that of the wild-type flagella. No significant difference in flagellar thickness was observed between the two, indicating that only one flagellin protein FlaA might be enough for synthesis of flagella with a normal thickness (data not shown). Additional experiments are necessary to completely characterize the physical nature of the thick *V. cholerae* flagella.

In conclusion, non-flagellated and thus, non-motile strains of *V. cholerae* were shown to be less virulent in a human volunteer study (Qadri *et al.*, 2007). Therefore, understanding the flagellar structure is important to elucidate the disease pathology of *V. cholerae* infections. Our results suggest that the *V. cholerae* flagellum could have a major structural defect associated with the lack of OMPs and such a deformity was most likely associated with high level release of flagellin proteins. More in-depth understanding of the roles of OMPs in *V. cholerae* motility will provide a better insight into the virulence mechanisms of this deadly enteric pathogen.

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