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Identification of OmpU of *Vibrio vulnificus* as a Fibronectin-Binding Protein and Its Role in Bacterial Pathogenesis

Sung Young Goo, Hyun-Ju Lee, Woo Hwang Kim, Kyu-Lee Han, Dae-Kyun Park, Hyun-Jung Lee, Sung Min Kim, Kun-Soo Kim, Kyu-Ho Lee, and Soon-Jung Park*  

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*Vibrio vulnificus* is a pathogenic bacterium that causes gastroenteritis and primary septicemia. To identify factors involved in microbial adherence to the host cells, we investigated bacterial proteins capable of binding to fibronectin, one of the main components comprised of the extracellular matrix of mammalian cells. A protein of ~35 kDa was purified from the extracts of *V. vulnificus* by its property to bind to immobilized fibronectin. This protein was identified as OmpU, one of the major outer membrane proteins of *V. vulnificus*. In binding assays using immobilized fibronectin, the number of ompU mutant cells bound to fibronectin was only 4% of that of wild-type cells bound to fibronectin. In addition, the exogenous addition of antibodies against OmpU resulted in a decreased ability of wild-type *V. vulnificus* to adhere to fibronectin. The ompU mutant was also defective in its adherence to RGD tripeptide (5% of the adherence of the wild type to RGD), cytoadherence to HEp-2 cells (7% of the adherence of the wild type to HEp-2), cytotoxicity to cell cultures (39% of the cytotoxicity of the wild type), and mortality in mice (10-fold increase in the 50% lethal dose). The ompU mutant complemented with the intact ompU gene restored its abilities for adherence to fibronectin, RGD tripeptide, and HEp-2 cells; cytotoxicity to HEp-2 cells; and mouse lethality. This study indicates that OmpU is an important virulence factor involved in the adherence of *V. vulnificus* to the host cells.

*Vibrio vulnificus* is a gram-negative pathogenic bacterium that is encapsulated, motile, and invasive. This pathogen causes primary septicemia, necrotizing wound infections, and gastroenteritis, especially in humans with a high alcohol drinking habit or hepatic diseases (24). Several virulence factors have been proposed for *V. vulnificus*: lipopolysaccharide (2, 19), capsular polysaccharide (42), a cytolytic hemolysin (10), an elastase (22), iron availability (43), and phospholipase A₂ (38). Genetic studies using knockout mutants of *V. vulnificus vvpA* (cytolytic protein) or *vvpE* (elastase) did not provide any evidence supporting that they were key virulence factors causing lethality of mice or death of HEp-2 cells (12, 35, 40). However, the cytolytic, but not the metalloprotease, was essential for causing damage in tissues of the infected mice (7). On the contrary, an important role of capsular polysaccharide was demonstrated by measuring attenuated mouse lethality of noncapsulated mutant *V. vulnificus* (45). Type IV pilin was also confirmed to be involved in the virulence of *V. vulnificus* via a genetic deletion of *pilD* or *pilA* gene (26, 27). In addition, motility was discovered to be a virulence determinant of *V. vulnificus* (15, 17).

Besides the structural apparatus, several transcriptional regulators were reported to be important for the pathogenesis of *V. vulnificus*. An alternative sigma factor, RpoS, was shown to be important for both the survival and virulence of this pathogenic bacterium (11, 29). An experiment employing in vivo-induced antigen technology identified the *hlyU* gene to be induced during the infection process of *V. vulnificus*, which encodes an activator for hemolysin production in *V. cholerae* (39). In that study, deletion of this gene was found to cause a dramatic decrease in bacterial cytotoxicity (16).

The first step in the microbial infection of host cells is mediated primarily by the interaction of the pathogen with connective tissues or epithelial cells (6). Fibronectin (FN) is considered the most important extracellular matrix (ECM) protein involved in cellular adherence (33), and it is one of the receptors on epithelial cells for bacterial adherence (5). Regarding the bacterial interaction with FN, gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* have been extensively investigated (reviewed in reference 34). In these microorganisms, FN-binding proteins (FNBP)s with conserved domains mediated bacterial adhesion to and invasion of host cells. Deletion analysis of FNBP genes of *S. aureus* localized primary FN-binding sites, such as repeats of 35 to 40 residues in the carboxyl-terminal part of the protein (8). Assays of invasion of *S. aureus* cells into human corneal epithelial cells indicated that inhibitors specifically blocking actin polymerization or tyrosine kinase of host cells abolished bacterial entry into host cells (13). This result suggested a model in which the invasion of *S. aureus* into host cells required the activation of a signal cascade including actin polymerization and tyrosine kinase and that FNBP may serve as a trigger for the activation of this signaling pathway.

In this study, we examined whether *V. vulnificus* may interact with the ECM, and specifically with FN, and demonstrated the adherence of *V. vulnificus* to immobilized FN. We then defined the nature of a bacterial surface protein(s) interacting with FN.
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
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<td></td>
</tr>
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<tr>
<td>MO6-24/O</td>
<td>Clinical isolate</td>
<td>41</td>
</tr>
<tr>
<td>DK1</td>
<td>ΔompU mutant of MO6-24/O</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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</tr>
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<td>pGEMT-Easy</td>
<td>Vector used for cloning of PCR product</td>
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<td>28</td>
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<td>Stratagene</td>
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<td>This study</td>
</tr>
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<td>pDM4</td>
<td>Suicide vector; OriRori Cm'</td>
<td>21</td>
</tr>
<tr>
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<td>pDM4 ΔompU</td>
<td>This study</td>
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<td>S. H. Choi</td>
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<tr>
<td>pSM1</td>
<td>pH0311 ompU'</td>
<td>This study</td>
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</table>

**MATERIALS AND METHODS**

Strains, plasmids, and culture cultivation. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains used for plasmid DNA preparation and the conjugal transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB plates containing 1.5% (wt/vol) agar. *V. vulnificus* strains were grown in LB medium supplemented with 2% (wt/vol) NaCl (LBS), unless stated otherwise. Ampicillin was added to the medium at 100 μg ml⁻¹ for the maintenance of plasmids in *E. coli* or at 300 μg ml⁻¹ for the selection of *V. vulnificus* exconjugants. All medium components were purchased from Difeo, and the chemicals and antibiotics were obtained from Sigma.

Assay of binding to the ECM. *V. vulnificus* strain MO6-24/O (41) was freshly grown up to an optical density at 600 nm (OD₆₀₀) of 1.0 in LBS at 30°C, harvested by centrifugation at 7,000 × g, and resuspended in phosphate-buffered saline (PBS) (150 mM NaCl, 17 mM Na₂HPO₄, 1 mM EDTA; pH 7.0). Ninety-six-well plates were coated with one of the ECM components (FN, collagen, or laminin; Sigma) at a concentration of 100 μg ml⁻¹ in PBS (4°C, 18 h). After blocking the plates with bovine serum albumin (BSA) (1% for 1 h at room temperature (RT), 1 × 10⁵ *V. vulnificus* cells were added to each of the coated wells. After being washed four times with PBS–1% BSA, bound bacteria were retrieved by resuspension in PBS-0.1% Triton X-100 at RT for 10 min and counted by plating onto LBS agar. The 96-well plate with BSA instead of anti-OmpU. Blood samples were also obtained from nonimmunized mice and then used as a control.

Assay of binding to RGD. Each well of 96-well culture plates was coated with 20 μg of RGD tripeptide (NH₂-Arg-Gly-Asp-COOH) (Takara) in PBS at 4°C for 18 h, blocked with PBS–1% BSA at RT for 1 h, and then washed three times with PBS. A total of 1 × 10⁵ cells of freshly grown *V. vulnificus* (OD₆₀₀ of 0.7) were added to each of the RGD-coated wells and incubated at RT for 30 min. After being washed four times with PBS–1% BSA, the bound bacteria were retrieved, serially 10-fold diluted, and then enumerated by plating onto LBS agar.

Treatment of *V. vulnificus* with OmpU-specific antibodies. To confirm the role of OmpU in bacterial binding to FN or to host cells as well as in bacterial cytotoxicity, wild-type *V. vulnificus* cells were precultivated with anti-OmpU (20–40 μg ml⁻¹) before being used for binding assays. As a control, wild-type *V. vulnificus* cells were treated with the same concentrations of mouse preimmune serum instead of anti-OmpU.

Construction of *ompU* mutant and complementation strains. Using SphI-SphI sites present in pDK1, a 1,630-bp DNA fragment of pDK1 was subcloned into pBluescript SK(−) (Stratagene) to yield pBS-DK1. After a 204-bp PstI DNA fragment was deleted in the *ompU* gene, the SphI-SphI DNA fragment of the *ompU*-deleted plasmid (1,426 bp) was then cloned into the suicide vector pDM4 (21). The resultant construct, pDM-dU, was used to mutate the *ompU* gene in wild-type *V. vulnificus* by allelic exchange to yield an *ompU* knockout *V. vulnificus* strain, DK1. Deletion of the PstI fragment within the *ompU* gene caused the loss of a portion of the protein (amino acid residues 15 to 82).

A 1,312-bp DNA was amplified from *V. vulnificus* genomic DNA using two 1.63-kb DNA fragment containing the *ompU* gene and a 513-bp upstream region from the genomic DNA of *V. vulnificus* MO6-24/O. This DNA was then cloned into the pGEMT-Easy vector (Promega) to produce pDK1. Using two EcoRI sites of pDK1 (one at position 252 from the start codon of *ompU* and the other in the multicloning site), a 791-bp DNA fragment containing the 5'-terminal portion of *ompU* was isolated and cloned into pTrHisB (Invitrogen), resulting in a tagging of OmpU with six histidines. After being transformed into JM109, recombinant OmpU was overexpressed and purified with a His-Bind kit (Novagen).

Six-week-old female BALB/c mice were immunized intraperitoneally with 10 μg of recombinant OmpU, which was emulsified 1:1 in Freund complete adjuvant. The animals wereboosted twice at 3-week intervals with the same amount of OmpU protein emulsified in Freund incomplete adjuvant. A week after the third immunization, blood samples of mice were pooled and used for further experiments as polyclonal antibodies against recombinant *V. vulnificus* OmpU (anti-OmpU). Blood samples were also obtained from nonimmunized mice and then used as a control.

Assay of binding to RGD. Each well of 96-well culture plates was coated with 20 μg of RGD tripeptide (NH₂-Arg-Gly-Asp-COOH) (Takara) in PBS at 4°C for 18 h, blocked with PBS–1% BSA at RT for 1 h, and then washed three times with PBS. A total of 1 × 10⁵ cells of freshly grown *V. vulnificus* (OD₆₀₀ of 0.7) were added to each of the RGD-coated wells and incubated at RT for 30 min. After being washed four times with PBS–1% BSA, the bound bacteria were retrieved, serially 10-fold diluted, and then enumerated by plating onto LBS agar.

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A 1,312-bp DNA was amplified from *V. vulnificus* genomic DNA using two
primers, ompU-F (5′-CTAGGAGCTCCACATCGAACATGTCTCAT-3′ [underlined bases indicate the SmaI site]) and ompU-R (5′-CTAGGAGCTCCAGATATGACAAATCTAT-3′ [underlined bases indicate the Smal site]), which contains an open reading frame and a 307-bp upstream region of the ompU gene. This DNA fragment was then cloned into the corresponding site of pJH0311 to produce pSM1. This ompU-containing plasmid was mobilized in V. vulnificus strain DK1 via conjugation. Strain DK1 carrying pH0311 was also constructed in the same manner to serve as a control.

**Western blot analysis.** Lysates of various V. vulnificus cells (wild type, DK1, DK1 with pH0311, and DK1 with pSM1) were prepared by resuspending the harvested cells in PBS and disrupting them with a sonicator. After centrifugation at 12,000 × g for 10 min, portions of the supernatants (50 μg of protein per well) were subjected to SDS-PAGE and then transferred onto a PVDF membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at RT and then treated with OmpU antibodies at 4°C overnight. Upon incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G, immunoreactive bands were visualized using an enhanced chemiluminescence system (Cell Signaling).

**Cell line adherence assay.** Adherence assays were performed with the HEp-2 cell line derived from human laryngeal epithelial cells (Korean cell line number 10023) in 24-well culture plates. Each well of the culture plates was seeded with 10^5 HEp-2 cells and grown overnight at 37°C in the presence of 5% CO2. After removing the medium and washing the cells twice with PBS, 1 ml of serum-free Dulbecco’s modified Eagle medium (Gibco-BRL) was added to the HEp-2 cells. Various V. vulnificus cells (wild type, DK1, DK1 carrying pH0311, and DK1 with pSM1) were grown at 30°C overnight in LBS broth. Cell monolayers were then inoculated in triplicate with 50 μl of the diluted bacteria to give a multiplicity of infection (MOI) of 10 and incubated at 37°C in 5% CO2 for 30 min. The monolayer was then washed five times with prewarmed PBS to remove nonadherent bacteria. Following the last wash, the HEp-2 cells were lysed with 0.1% Triton X-100 treatment for 15 min. The bacteria were recovered from these cells with PBS, serially 10-fold diluted, and then plated onto LBS agar.

**Cytotoxicity assays.** Cytotoxicity of V. vulnificus to HEp-2 cells was measured using the Cytotox 96 Non-Radioactive Cytotoxicity assay kit (Promega). This cytotoxicity kit measures the lactate dehydrogenase (LDH) activity released into the culture medium by lysed cells. To measure the total LDH activity of the cell lines used in the assays, Triton X-100 was added to a final concentration of 1.0% (vol/vol) to lyse the host cells. The LDH activity was then determined by colorimetric assay according to the manufacturer’s instructions and is represented as a percentage of LDH activities relative to the total LDH activities of the cells treated with 1% Triton X-100.

As a second assay to measure the cytotoxicity of V. vulnificus, the viability of HEp-2 cells was also checked using propidium iodide (PI), which stains nucleic acids of dead cells with disrupted membranes. Flow cytometric analysis to estimate the percentage of cells stained with PI was performed on at least 5,000 cells from each sample by fluorescence-activated cell sorting (FACScan; BD Biosciences).

**LD50 determination.** For 50% lethal dose (LD50) determinations, specific-pathogen-free, 7-week-old, female ICR mice were used without pretreatment with iron-dextran. Cultures of bacterial strains grown overnight in LB medium with 0.86% (wt/vol) NaCl were freshly cultivated in the same medium up to an OD600 of 0.7, harvested, washed once in PBS, and then resuspended in PBS-0.01% gelatin. One hundred microliters of serial dilutions of the bacterial suspension was then injected intraperitoneally into six mice per dilution group. The numbers of dead mice were determined 24 h after the injection, and the LD50 was calculated using the equation described previously by Reed and Muench (32).

**Statistical analyses.** Results were expressed as the means ± standard deviations from three independent experiments. Statistical analysis was performed using Student’s t test (SYSTAT program, SigmaPlot version 9; Systat Software Inc.). Differences were considered significant if P values were <0.05. Data with P values of <0.01 are indicated with two asterisks, whereas data with P values between 0.01 and 0.05 are indicated with one asterisk.

**RESULTS**

**Binding of V. vulnificus to ECM components.** We examined the ability of V. vulnificus to adhere to three components of ECM, collagen, fibronectin, and laminin (Fig. 1). In contrast to the low number of bacterial cells bound to the coated BSA (3.4% of the added bacteria), higher numbers of bacteria were retained on coated fibronectin and collagen. However, the percentage of bacteria that bound to laminin was similar to
that of bacteria that adhered to BSA (4.1% of the added *V. vulnificus* cells). Despite the higher bacterial affinity for collagen (13% of the added bacteria), the number of bound bacteria was not statistically different from that of bacteria bound to BSA. Numbers of bacteria that bound to FN (18% of the added bacteria) were significantly higher (*P* < 0.002) than the numbers of bacteria that bound to BSA.

Binding of *V. vulnificus* to immobilized FN was further examined in subsequent experiments. The numbers of bacteria that bound to an FN-coated surface increased in a dose-dependent manner (Fig. 2). The percentage of bacteria that bound to FN was increased up to 14% at 100 µg ml⁻¹ of FN. On the other hand, less than 3% of added bacteria were retained on the surface coated with BSA regardless of the amount of BSA used.

**Identification of the OmpU protein as a fibronectin-binding protein of *Vibrio vulnificus***. Binding of *V. vulnificus* to FN led us to identify a bacterial protein involved in this process. A protein of about 35 kDa was retrieved from lysates of *V. vulnificus* MO6-24/O by its affinity for FN-coated surfaces (Fig. 3). The sequence of the first 21 amino acids at the amino terminus of the isolated protein was AELYNQDGTSLDMGGRAEARL. A TBLAST search using this partial sequence showed complete identity to the OmpU proteins of both *V. vulnificus* strain CMPC6 (GenBank accession number AE016802.1) and *V. vulnificus* strain YJ016 (accession number BA000037.2). In the analysis of the proteins isolated from FN-binding assays, we could not observe the protein bands corresponding to FN (200–250 kDa [plasma FN] or 550 kDa [cellular FN]) on a PVDF membrane, possibly due to the low efficiency of blotting of these proteins with high molecular weights.

**Role of OmpU protein in binding of *V. vulnificus* to the immobilized FN and to immobilized RGD tripeptide**. The function of OmpU in the FN binding of *V. vulnificus* was assessed using an *ompU* knockout mutant. The *V. vulnificus* *ompU* mutant was made by removing a PstI DNA fragment of 204 bp, which is present within an open reading frame of the *ompU* gene. The mutant results in a loss of a portion of the amino acids from residues 15 to 82 of OmpU.

*ompU* mutant strain DK1 was verified by Western blot analysis using anti-OmpU (Fig. 4A). While OmpU was detected in lysates of wild-type *V. vulnificus* MO6-24/O, the immunoreactive band was not present in lysates of DK1. The intact OmpU gene was added back to *ompU* mutant strain *V. vulnificus* DK1 as a pH0311-based plasmid, pSM1. Strain DK1, containing pH0311, was also constructed as a control strain. Expression of the OmpU protein in the complemented strain was also shown in Western blots using anti-OmpU, whereas the OmpU protein was not detected in the control strain, DK1 harboring pH0311 (Fig. 4B).

The ability of this *ompU* mutant to bind to FN was compared with that of the wild-type strain (Fig. 5A). While a significant
portion of the added wild type was bound to FN in a dose-dependent manner (15%), the ability to bind to FN was significantly disrupted in the ompU knockout mutant; that is, 55% of the added mutant bacteria were bound to the FN-coated surface.

Evidence for the involvement of OmpU of V. vulnificus in FN binding was strengthened by an additional experiment using anti-OmpU. Preincubation of wild-type V. vulnificus with anti-OmpU at concentration of 20 μg ml⁻¹ significantly decreased the bacterial binding of 3.3% of the added bacteria to FN (Fig. 5B). On the other hand, pretreatment of wild-type V. vulnificus with the same concentration of mouse preimmune serum did not result in any alteration of the bacterial binding to FN (13%).

The FN-binding ability of the ompU-complemented strain, DK1(pSM1), was also examined (Fig. 5A). The ability of the bacteria to adhere to FN was fully restored when the intact ompU gene was added to the ompU mutant strain. As expected, the presence of a vector plasmid, pJH0311, did not alter the inability of the ompU knockout strain to adhere to FN.

It is well known that FN-binding proteins bind to FN by specific interactions with the RGD repeat of FN (9). When we examined whether V. vulnificus could bind to immobilized RGD tripeptide, we found that a significant portion of V. vulnificus cells (55% of the added bacteria) was bound to the RGD tripeptide (Fig. 6A). On the other hand, the ability of bacteria to adhere to RGD was almost abolished in the case of ompU knockout mutant DK1 (2.7% of the added bacteria). Wild-type V. vulnificus was treated with 20 μg ml⁻¹ of anti-OmpU or mouse preimmune serum and then tested for binding to RGD tripeptides (Fig. 6B). Twenty-four percent of V. vulnificus cells adhered to RGD when they were treated with preimmune serum by Student’s t test. Data with P values of <0.01 are indicated with two asterisks, whereas data with P values of between 0.01 and 0.05 are indicated with one asterisk.

Role of OmpU in cytoadherence of V. vulnificus. Based on the observation that OmpU may serve as a ligand for FN, we extended our experiments to reveal the function of this protein in interactions with host cells. The first assay that we performed was an examination of the role of OmpU in bacterial
adherence to the HEp-2 epithelial cell line (Fig. 7A). When the ratio of bacteria to HEp-2 cells was 10:1, the percentage of adhered bacteria of the initially added bacteria was 2.7% in the case of the assay of HEp-2 cells with wild-type *V. vulnificus*. In the case of the assay with the *ompU* mutant, the portion of the bacterial cells that adhered to the HEp-2 cells was quite low, e.g., 0.18% of the added bacteria.

The role of OmpU in the cytoadherence of wild-type *V. vulnificus* was confirmed using anti-OmpU (Fig. 7B). Wild-type *V. vulnificus* cells were treated with either 20 or 40 μg ml⁻¹ of anti-OmpU prior to the adherence tests while the ratio of bacteria to HEp-2 cells was kept at 10:1. As a control, wild-type *V. vulnificus* cells were also incubated with mouse preimmune serum at the same concentrations and used for the adherence tests. In the case of wild-type *V. vulnificus* cells preincubated with anti-OmpU, they showed decreased levels of cytoadherence in a dose-dependent manner, down to 0.77% of the anti-OmpU-treated cells. In contrast, 2.6 or 2.9% of the added *V. vulnificus* cells adhered to the cell lines when they were treated with 20 or 40 μg ml⁻¹ preimmune serum, respectively.

In addition, the cytoadherence of strain DK1 to HEp-2 cells was fully recovered to wild-type levels when it carried pSM1, an *ompU*⁺ plasmid, whereas the introduction of pJH0311 to strain DK1 did not cause any change in bacterial adherence to HEp-2 cells (Fig. 7A).

**Role of OmpU in cytotoxicity of *V. vulnificus***. In a subsequent experiment, we asked whether the defects in FN binding as well as in cytoadherence of the *ompU* mutant could affect bacterial virulence toward host cells. Incubation of wild-type *V. vulnificus* cells at an MOI of 50 or 100 resulted in 37% or 79% cytotoxicity of host cells, respectively, when determined by LDH release assay (Fig. 8A). On the other hand, the *V. vulnificus* *ompU* mutant was significantly less cytotoxic than wild-type *V. vulnificus* (14% and 32% at MOIs of 50 and 100, respectively).

Upon incubation with either wild-type or *ompU* *V. vulnificus*, the viability of HEp-2 cells was also checked by staining the dead cells with PI (Fig. 8B). Without the addition of *V. vulnificus*, 4.0 to 4.4% of HEp-2 cells were stained with PI, whereas the percentages of PI-stained cells exposed to wild-type *V. vulnificus* increased to 18 and 42% at MOIs of 10 and 20, respectively. When HEp-2 cells were incubated with the *V. vulnificus* *ompU* mutant at MOIs of 10 and 20, only 5.1 and 9.4% of HEp-2 cells, respectively, were stained with PI. These values are significantly less than those found with the wild type.

In the subsequent experiments, we examined whether attenuated phenotypes of strain DK1 in cytotoxicities to HEp-2 cell lines could be recovered to the wild-type phenotype by introducing pSM1. In the assays for measuring bacterial cytotoxic-
FIG. 8. Role of the OmpU protein of *Vibrio vulnificus* in bacterial cytotoxicity to HEp-2 cells. (A) Determination of cytotoxicity of various *V. vulnificus* strains by estimating the activity of LDH. Using a CytoTox96 assay kit, LDH released from the HEp-2 cells was measured upon incubation with wild-type *V. vulnificus* (open bars), *ompU* mutant DK1 (black bars), DK1 carrying pJH0311 (gray bars), or DK1 carrying pSM1 (hatched bars). HEp-2 cells (1 × 10⁵) were incubated with *V. vulnificus* cells for 30 min at two different MOIs, 50 and 100. Each assay was performed in triplicate and repeated three times. The data are shown with error bars, which are standard deviations from three independent experiments. Asterisks indicate enzyme activities that were significantly different (*P* < 0.01) from that of wild-type *V. vulnificus* cells by Student’s *t* test. (B) Determination of the cytotoxicities of various *V. vulnificus* strains by staining with PI. The percentages of dead HEp-2 cells during incubation with wild-type *V. vulnificus* (open bars), *ompU* mutant strain DK1 (black bars), DK1 carrying pJH0311 (gray bars), or DK1 carrying pSM1 (hatched bars) were measured at two different MOIs, 10 and 20. HEp-2 cells not exposed to *V. vulnificus* were also stained with PI (dotted bars). Error bars represent the means ± standard deviations from three independent experiments. Asterisks indicate percentages of PI-stained cells that were significantly different (*P* < 0.01) from that of wild-type *V. vulnificus* cells by Student’s *t* test.

DISCUSSION

The capability of bacterial adherence to the host cells is an important virulence factor for many pathogens, including *V. vulnificus*. The severe and rapid cytopathological characteristics of *V. vulnificus* infection make this organism a model system to investigate host-pathogen interactions. In adhesion assays using immobilized ECM components, *V. vulnificus* showed an ability to bind to FN (Fig. 1), suggesting that it may possess a surface protein(s) that interacts with this host component. In a subsequent experiment to isolate the adherence factor of *V. vulnificus* binding to FN, the OmpU protein, one of the outer membrane proteins, was identified (Fig. 3).

OmpU has been extensively studied in *V. cholerae*, a pathogen that colonizes the human intestine and produces cholera disease (25). The expression of *ompU*, encoding outer membrane porin in *V. cholerae*, is positively regulated by ToxR, the key regulator of cholera toxin production and other virulence determinants (4). Through a genetic construction of *V. cholerae* overexpressing OmpT, the other major outer membrane porin, the ToxR-dependent modulation of two outer membrane porins, OmpU and OmpT, was found to be important for the expression of virulence factors and intestinal colonization by *V. cholerae* (30). Interestingly, *V. cholerae* OmpU was previously found to be an FN-binding protein and was sug-

TABLE 2. LD₅₀s of *Vibrio vulnificus*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>No. of dead mice/no. of total mice for no. of injected bacteria</th>
<th>LD₅₀ (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO6-24/O</td>
<td><em>ompU</em></td>
<td>3.4 × 10⁵</td>
<td>3.4 × 10⁵</td>
</tr>
<tr>
<td>DK1</td>
<td>Δ<em>ompU</em></td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>DK1(pJH0311)</td>
<td>Δ<em>ompU</em></td>
<td>1.5</td>
<td>5.1</td>
</tr>
<tr>
<td>DK1(pSM1)</td>
<td><em>ompU</em></td>
<td>4.6</td>
<td>6.6</td>
</tr>
</tbody>
</table>

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gusted to be a potential adherence factor in *V. cholerae* (37). In that study, OmpU protein selectively bound to FN and to an arginine-glycine-asparagine (RGD) tripeptide. Subsequent studies, however, indicated that OmpU was not involved in the adherence of *V. cholerae* to the brush border of the rabbit small intestine (25, 31). In contrast, OmpU was reported to be an important colonization factor in *Vibrio fischeri* to establish mutualistic symbiosis within a light organ of its squid host, *Euprymna scolopes*; however, its affinity for FN was not reported (1). It is likely that OmpU proteins in *Vibrio* spp. may function as a component of the adhesion apparatus via their affinity for FN. Sequence comparison of OmpU proteins indicates that this protein is conserved in various *Vibrio* spp. including *V. cholerae*, *V. parahaemolyticus*, *V. fischeri*, *V. splendidus*, and *V. vulnificus*. The identities of amino acid sequences range from 59 to 76% among OmpU-homologous proteins.

Our study using an *ompU* knockout *V. vulnificus* mutant and antibodies against OmpU clearly shows that the OmpU protein of *V. vulnificus* is involved in FN binding (Fig. 4). Adherence and cytotoxicity assays (Fig. 5, 6, and 7) demonstrated that *V. vulnificus* cells interact with the host cell epithelium via a direct interaction between bacterial OmpU and FN of the host cells at the early stage of infection. Failure of the *ompU* mutant of *V. vulnificus* to adhere to host cells apparently results in defects in bacterial cytotoxicity against host cells. In gram-positive bacteria, several FNBPs had been reported as major virulence factors: FNBP1 and FNBP2 for *S. aureus* (14, 36), Sfb1 for *Streptococcus pyogenes* (23), and CsaH for *Streptococcus gordonii* (18). A current model for the function of these FNBPs suggested that the binding of FNBP with multiple FNs resulted in the localization of integrin binding sites, which induce the clustering of integrins in the membrane of host cells (34). The clustering of integrin in host cells may trigger the phosphorylation of tyrosine kinase and actin rearrangement, which are essential for the internalization of bacteria into host cells (13). In the case of *Streptococcus pyogenes*, two distinct cellular mechanisms were proposed for Sfb1-negative and Sfb1-positive strains with respect to the involvement of integrin in bacterial invasion (23). In contrast, nothing is known about the exact mechanism(s) by which OmpU facilitates the pathogenesis of *V. vulnificus*, and further investigations should be performed to evaluate the contribution of the OmpU-FN interaction to the processes causing disease in humans by *V. vulnificus*.

**ACKNOWLEDGMENT**

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