VP1686, a *Vibrio* Type III Secretion Protein, Induces Toll-like Receptor-independent Apoptosis in Macrophage through NF-κB Inhibition*

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*Vibrio parahaemolyticus*, causative agent of human gastro-intestinal diseases, possesses several virulent machineries including thermostable direct hemolysin and type III secretion systems (TTSS1 and -2). In this report, we establish that TTSS1-dependent secretion and translocation of a *V. parahaemolyticus* effector protein VP1686 into the cytosol induces DNA fragmentation in macrophages. We performed yeast two-hybrid screening to identify the molecules involved in VP1686-mediated cell death pathways and showed that nuclear factor RelA p65/NF-κB physically interacts with VP1686. To understand the impact of this interaction on the NF-κB DNA binding activities in infected macrophages, we analyzed a series of deletion mutants for the TTSS and its effector protein VP1686 that have known functions in cellular responses to apoptosis, transcriptional regulation. Our results suggest an important role for *Vibrio* effector protein VP1686 that activate a conserved apoptotic pathway in macrophages through suppression of NF-κB activation independent of Toll-like receptor signaling.

When encountered with bacterial pathogens, multicellular organisms raise a series of defense mechanism. To ensure the survival, pathogenic bacteria have also evolved sophisticated invasion strategies that involve neutralization of host defense through inhibition of innate immune system, phagocytosis, or induction of apoptosis in macrophages. A broad range of bacterial pathogens, including Gram-negative bacterium *Vibrio parahaemolyticus*, uses a type III secretion system (TTSS) to deliver bacterial effector proteins into the host cell cytosol where they disrupt the signaling network (1–3). The TTSS machinery is a needle-like structural complex formed by about 20 proteins, and its components are highly conserved among the bacterial species (4). Recently genome sequencing of the clinical *V. parahaemolyticus* strain revealed that the strain has two gene clusters, TTSS1 and TTSS2, each encoding distinct type III secretion systems (5). Of the two TTSS encoding gene clusters of *V. parahaemolyticus* strain RIMD2210633, TTSS1 is almost similar to those of *Yersinia* spp. and *Pseudomonas aeruginosa* in the number of genes, their order, and in the identity of each encoded protein. To date, four TTSS1-secreted effector proteins of *V. parahaemolyticus* (VP1686, VP1683, VP1680, and VPA450) have been identified by using a two-dimensional gel electrophoresis, but none of them showed any significant homology to known effector proteins of other bacteria within the TTSS1 region (6).

In vertebrates, the main function of the innate immune system is to recognize the presence of pathogen-associated molecular patterns on invading microbes and initiate downstream signal from Toll-like receptors (TLRs), which lead to the expression of inflammatory response-related genes (7). The transcription factor nuclear factor κB (NF-κB) is a critical mediator of TLR signaling that controls the synthesis of cytokines, adhesion molecules, and other anti-apoptotic factors such as inhibitor of apoptosis protein, tumor necrosis factor receptor-associated factor, and Bcl-2 families to ensure cellular survival by prevention of cell death (8–11). Up-regulation of antiapoptotic protein synthesis through NF-κB activation is also essential for host survival under versatile stress-induced conditions such as bacteria-faced macrophages. Thus, it is plausible, given the function of NF-κB to cell survival, that

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2 The abbreviations used are: TTSS, type III secretion systems; MyD88, myeloid differentiation primary response protein 88; TRIF, TIR domain-containing adapter-inducing interferon β; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; TLR, Toll-like receptors; PI, propidium iodide; FITC, fluorescein isothiocyanate; Fic, filamentation induced by σAMP.
improper activities of this protein may also be involved in bacteria-induced macrophage apoptosis.

Our previous study on DNA fragmentation patterns in HCT116 cells revealed that the induction of apoptosis by *V. parahaemolyticus* in these cells requires functional TTSS1 (1). But the mechanism by which TTSS1 induces apoptosis in HCT116 cells remains poorly defined. In this study we show that VP1686 is an effector protein of *V. parahaemolyticus* injected by type III secretion system 1 into the cytoplasm and induces apoptosis in both cultured and thioglycollate-elicited peritoneal macrophages. VP1686 binds with RelA p65/NF-κB in yeast and inhibits the DNA binding activity of NF-κB in macrophages. On the basis of these results, we suggest that inhibition of NF-κB is sufficient to sensitize infected-macrophages to death by preventing induction of NF-κB targeted gene expression related to antiapoptotic function.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—All the *V. parahaemolyticus* and *Vibrio* species strains used in this study were obtained from the Laboratory for Culture Collection, Research Institute for Microbial Diseases, Osaka University. *V. parahaemolyticus* strain RIMD2210633 (KP-positive, serotype O3:K6) was used for the construction of deletion mutants and for functional studies. *Escherichia coli* DH5α and SM10Apir (12) strains were used for the general manipulation of plasmids and the mobilization of plasmids into *V. parahaemolyticus*, respectively. The bacteria were cultured at 37 °C with shaking in Luria-Bertani (LB) medium (for *E. coli*) and LB supplemented with 3% NaCl (for *V. parahaemolyticus*). Thiosulfate citrate bile salts sucrose agar (Nissui, Tokyo, Japan) was used for the screening of mutant strains. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (5 μg/ml).

**Cell Lines**—RAW264.7 cells were grown as monolayer in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 10 units/ml penicillin G. Mouse peritoneal macrophages were collected by peritoneal lavage with Hanks’ balanced salt solution at 3 days after intraperitoneal injection of 2 ml of 4% sterile thioglycollate into 8–12-week-old mice. Peritoneal macrophages were cultured in RPMI 1640 medium with 10% fetal bovine serum, 100 μg/ml streptomycin, and 10 units/ml penicillin G.

**VP1686 Complementation**—The VP1686 gene (operon) containing a putative promoter region from the KXV-237 (RIMD2210633) strain was amplified by PCR using the following oligonucleotide primers 5'-GAGTAGGGGATCCCGCCTGCAAA-A3' and 5'-AATACCAACGTCGAATCAC-3'. The amplified fragment was cloned into a pT7blue T vector and digested with BamHI and Sall. The digested fragment was then cloned into the pSA19Cm-MCS (13). The plasmid was introduced into KX-V237 VP1686 deletion mutant by electroporation (1.5 kV, 1000 ohms, 25 microfarads).

**Infections of MyD88−/−, TRIF−/− Macrophages**—MyD88 and TRIF knock-out mouse and Balb/c mice were injected with 4% thioglycollate 3 days before harvesting peritoneal macrophages. Approximately 2 × 10^5 peritoneal macrophages were seeded into a 10-mm dish and infected as described above.

**DNA Fragmentation**—Both fragmented DNA and high molecular weight intact genomic DNA were extracted from 2 × 10^6 cells using the suicide-Track™ DNA ladder isolation kit (Oncogene, Madison, WI). 1.5% agarose gel electrophoresis was followed by ethidium bromide staining.

**Annexin V-FITC Apoptosis Detection Assay**—The cells were harvested and infected with *V. parahaemolyticus* as described above and washed three times with phosphate-buffered saline. Staining was carried out using the annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA). Briefly, 2 × 10^5 cells were resuspended in 1× binding buffer and incubated with annexin V-FITC and propidium iodide (PI) for 5 min in darkness at room temperature. Annexin V binding was analyzed by FACScan cytometer (BD Biosciences) equipped with a FITC signal detector FL1 (excitation = 488 nm, green) and PI staining by the phycoerythrin emission signal detector FL2 (excitation = 585 nm, red). The percentage of apoptotic cells was calculated from the total (10^4 cells) using FlowJo 4.5.2 software.

**Cytotoxicity assays**—RAW 264.7 macrophages (2 × 10^6 cells) were seeded onto a 96-well plate and incubated overnight at 37 °C. Before infection with the bacteria, cells were washed with phosphate-buffered saline, pH 7.2, and further incubated with Dulbecco’s modified Eagle’s medium without phenol red and antibiotics. The release of lactate dehydrogenase (LDH) into the medium was assayed using the CytoTox96 nonradioactive cytotoxicity kit (Promega) according to the manufacturer’s instructions. At 4 h post-infection with bacteria, the supernatants were collected, and the release of LDH was quantified.

**Electrophoretic Mobility Shift Assay**—RAW macrophages were washed with cold phosphate-buffered saline and pelleted. The cells were lysed in buffer X + bovine serum albumin (BSA; 100 mM Tris-HCl, pH 8.5, 250 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 1 μg/ml aprotinin, 2 mg/ml BSA). Whole cell protein lysates were solubilized in loading buffer, subjected to SDS-PAGE, and transferred to nitrocellulose followed by incubation with the antibodies as mentioned in the figure legends.


**RESULTS**

**Identification of VP1686**—Recent genome sequencing of the clinical *V. parahaemolyticus* strain RIMD2210633 identified by two-dimensional gel electrophoresis (6). Using the proposed amino acid sequence of VP1686 (5), our conserved domain search using protein-protein BLAST detected that VP1686 belongs to the family of proteins called Fic (filamentation growth). A domain alignment comparing the HPFXXGN region of VP1686 and 24 other family members (the most important 9 are shown). Residues that compose Fic are highlighted by underlines. The specificities of the shifted band were confirmed by adding antibodies specific for p65 NF-κB.

**Microarray Gene Chip Analysis**—RAW cells were infected with TTSS deletion mutants of *V. parahaemolyticus* (m.o.i. 2) for 2 h. Total RNA was extracted (RNase kit, Qiagen), and double-stranded cDNA was synthesized from 5 μg of total RNA using the Superscript system (Invitrogen) primed with a T7-(dT)24 primer. To prepare biotin-labeled cRNA from this cDNA, an *in vitro* transcription reaction was performed in the presence of T7 RNA polymerase and biotinylated ribonucleotides (Enzo Diagnostics). The cRNA product was purified in the presence of T7 RNA polymerase and biotinylated ribonucleotides (Enzo Diagnostics). The chips were washed and scanned with a GeneArray scanner (Affymetrix). The color intensity of gene expression was generated with the R (Version 2.2.1) and Bioconductor software.

**Results**

**Inhibition of DNA Fragmentation in V. parahaemolyticus-infected macrophages is TLR-independent and Requires Both TTSS1 and VP1686**—In our previous studies, we have shown that one of the characteristic effects of TTSS1 is cytotoxicity to eukaryotic cells (1, 15). We performed DNA fragmentation and annexin V staining assays of macrophages infected with mutant *V. parahaemolyticus* strain carrying deleted genes that encode TTSS system 1 and 2 as well as individual secretion proteins to determine whether any of them play a role in initiating programmed cell death in macrophages. Primarily, mutant strains lacking TTSS1, TTSS2, and each of the three proteins encoded by VP1686, VP1680, VPA450 were exposed to RAW macrophages (m.o.i. 2) (Table 1). Total DNA (genomic and fragmented) was prepared from infected cells after 4 h. Electrophoretic patterns indicate the presence of oligonucleosomal DNA at room temperature and loaded on a native 5% polyacrylamide gel. The DNA-protein complex was visualized by autoradiography. The specificities of the shifted band were confirmed by adding antibodies specific for p65 NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA).

**VP1686 Induces Macrophage Apoptosis through NF-κB Inhibition**

**FIGURE 1.** Structure and expression of TTSS1-dependent effector protein VP1686. A, RAW 264.7 cells (2 × 10⁶) cultured in antibiotic-free Dulbecco’s modified Eagle’s medium were infected with wild type (WT), TTSS1-deleted, and TTSS2-deleted strains of VP at a m.o.i. of 2 for 3 h. Whole cell RAW lysates (WCE) and tissue culture medium were subjected to Western blot analysis using VP1686-specific polyclonal antibodies. B, predicted amino acid sequence of VP1686. C, conserved domain search using the amino acid sequence above identified that the VP1686 protein belongs to a family of proteins carrying a central conserved motif HPFXXGN called Fic. D, domain alignment comparing the HPFXXGN region of VP1686 and 24 other family members (the most important 9 are shown). Residues that compose Fic are highlighted by underlines.
length DNA fragmentation (≤190 bp) in cells infected with TTSS2-deleted strain but not in uninfected cells or cells infected with TTSS1-deletion mutants (Fig. 2, A and B). Neither heat-killed bacteria nor bacteria-free cultural supernatant of 4-h post-inoculation were able to induce DNA fragmentation in RAW cells (data not shown). These results indicate that intact live bacteria and its TTSS1 components (not TTSS2) are involved in the apoptosis of macrophages and, thus, the cytotoxic activity of the parental strain.

To test whether the phenotype described above resulted from the specific function exerted by TTSS1, RAW cells were infected with TTSS1-containing but TTSS2-deleted mutants of V. parahaemolyticus for 1.5 h. No DNA fragmentation was seen in these cells in this stage. We then washed the cells extensively to remove the extracellular bacteria or kill bacteria by incubating cells in fresh medium with 100 μg/ml gentamycin for 2, 3, and 6 h. In the absence of any surrounding live bacteria, apoptotic DNA fragmentation started to appear in cells 3 h after gentamycin treatment, indicating that the removal/killing of extracellular bacteria was unable to halt the signaling events initiated in these cells in the first 1.5 h (Fig. 2C). We hypothesize that once V. parahaemolyticus injects VP1686 inside the host cell cytoplasm, subsequent action of VP1686 were irreversible. With the help of Western blot analysis in an earlier experiment, we have indeed confirmed the presence of TTSS1-dependent VP1686 translocation in apoptotic RAW whole cell extract and in cell culture supernatant (Fig. 1A). Several previous studies demonstrated that activated TLRs could act as a potent inducer of apoptosis in macrophages that encounter a bacterial pathogen (16, 17). To determine whether TLR signaling could play a role in the mechanism of apoptosis induction by V. parahaemolyticus we investigated DNA fragmentation pattern in primary mouse peritoneal macrophages that are deficient for functional TLR adapters MyD88 (myeloid differentiation primary response protein 88) and TRIF (TIR domain-containing adapter inducing interferon β). MyD88 is a central adapter shared by almost all TLRs (18), and TRIF is used in TLR4 signaling activated by bacterial LPS independent of MyD88 (19). Surprisingly, both MyD88−/− and TRIF−/− macrophages underwent apoptosis upon infection with TTSS1-containing mutant (Fig. 2D) Thus, signaling events originated from TLR due to bacterial recognition is not essential for VP1686-induced macrophage apoptosis.

**TABLE 1**

| Strain/plasmid | Genotype or relevant phenotype | Source |
|----------------|-------------------------------|--------|
| V. parahaemolyticus strains | | |
| RIMD2210633 | Clinical isolate: tdhA+ / tdhS+ | 25 |
| POR-1 | Both tdh(AS) deleted from RIMD2210633 | 15 |
| ΔTTSS1 | POR-1ΔvcrD-1 | 2 |
| ΔTTSS2 | POR-1ΔvcrD-2 | 2 |
| VP31686 | POR-1ΔVP1686 | 6 |
| VP31680 | POR-1ΔVP1680 | 6 |
| VP4A50 | POR-1ΔVP4A50 | 6 |
| VP1686 complimented | POR-1ΔVP1686 + VP1686 | This study |

V-FITC and PI followed by flow cytometry. Fig. 3 shows that in RAW cells, when infected with mutant strains that lack either TTSS1 or its effector VP1686, only 7.6 and 3.9% cells were stained positive (right-bottom quadrant) for phosphatidylinerine, a marker of early stages of apoptosis. Almost an equal number of the apoptotic cells were stained for asynchronously grown uninfected cells (5.7%), which could be the cells committing natural cell death without any external induction. Interestingly, a considerable increase in apoptotic cells was found when macrophages were infected with the mutant strains lacking TTSS2, VP1680, or VPA450, but TTSS1 and VP1686 were retained (39.6, 37.8, and 26.2%, respectively). These results strongly suggest that the remaining secretory components such as VP1686 and its injection machinery TTSS1 in the parental strain might be the principal apoptosis inducer in macrophages.

**FIGURE 2.** DNA fragmentation analysis showing induction of apoptosis. A and B, agarose gel (1.5%) electrophoresis of DNA isolated from RAW cells either uninfected or infected with TTSS1-deleted and TTSS2-deleted strains of VP at a m.o.i. of 2 for 4 h. Con, control. C, RAW macrophages remained untreated or were infected with TTSS1-positive mutant (TTSS2-negative) for 1.5 h. Cells were then washed with fresh medium containing gentamycin (100 μg/ml) to remove external bacteria. Onset of apoptosis was monitored 1, 2, 3, and 4 h after or post washing (PW) by analyzing the initiation of DNA fragmentation in these cells. D, peritoneal macrophages (PEC) elicited from the indicated mice remained untreated or infected with TTSS1-positive mutant (TTSS2-negative) for the indicated period of time. Onset of apoptosis was monitored for a 1-h interval after infection by analyzing DNA fragmentation pattern in these cells.
release from the RAW macrophages, we see the changes in reversion of its cell death ability due to VP1686 complementation. LDH, a stable cytosolic enzyme, was shown to be released upon cell lysis during the later stages of apoptosis as well as in early stages of necrosis (20). To confirm the successful genetic complementation, we detected the expression of VP1686 protein by Western blot analysis in previously VP1686 non-producing strains (Fig. 4A). Consistent with VP1686 carrying wild type strains, as shown in Fig. 4B, the wild type and the VP1686-complemented strains showed increased and almost indistinguishable levels of macrophage cell death (75 and 85%, respectively) after 4 h of infection (m.o.i. 2). The LDH experiment also supports our existing annexin V staining result that both the TTSS1-deleted mutant and VP1686-deleted mutants showed considerable decreases in cellular cytotoxicity (20–25%). Interestingly, macrophages when incubated with LPS for 8 h (50 ng/ml) did not show any increase in cell death. A restored level of LDH release attributed to VP1686-complemented strains prompted us to conclude that this protein plays a specific role in macrophage cell death.

VP1686 Interacts with NF-κB p65 in Yeast and Suppresses DNA Binding Activity of NF-κB in Macrophages—Unlike several other bacterial infection-induced apoptosis in macrophages, the mechanism of VP1686-induced apoptosis was not dependent on cell cycle arrest, caspase-1 activity (data not shown), and TLR-mediated signaling. In an effort to understand the mechanism of VP1686-induced apoptosis in macrophages, we employed the yeast two-hybrid method to identify VP1686-interacting protein(s) by using full-length VP1686 as bait. From a screen of ~4×10⁵ yeast transformants, 14 cDNA clones scored positive for reporter gene activities. Sequence analysis revealed that two of these clones encoded a similar portion of NF-κB. To further explore this interaction, full-length VP1686, full length of another TTSS1-dependent secretor protein VP1680, the N terminus of VP1686, and the C terminus of VP1686 were studied in the yeast two-hybrid systems.
VP1686 Induces Macrophage Apoptosis through NF-κB Inhibition

NF-κB is an important transcriptional regulator of inducible expression of numerous genes involved in apoptosis, inflammation, and innate immune response. To investigate whether *V. parahaemolyticus* induces NF-κB DNA binding activity consistent with *Entero-pathogenic Eschericia coli*, *Yersinia*, and *Salmonella*, RAW cells were challenged with *V. parahaemolyticus* for 2 h. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assays using consensus oligonucleotide NF-κB probe. Uninfected cells served as a negative control, whereas macrophage incubated with LPS for 2 h was used as a positive control. Fig. 5B shows that the VP1686 inhibits DNA binding activity of the NF-κB because as a result of TTSS1 deletion from the strain that no longer can secrete VP1686, NF-κB was able to bind with DNA in macrophages. As expected, NF-κB activation was severely impaired in macrophages infected with TTSS2-deleted strain, which contains functional TTSS1. Similar to TTSS1 mutant, VP1686-deleted strain also failed to block NF-κB activation. In addition, we have found that upon re-introduction of the VP1686 gene in the VP1686-deleted strain by genetic complementation, the capability to suppress activation of NF-κB was restored. The specificity of the band was confirmed by a shift by treatment with anti-p65 antibodies (Fig. 5C). Together, the analysis of the different mutant *Vibrio* strains indicates that VP1686 impairs NF-κB activation and subsequently mediates apoptosis in macrophages.

**Differential Gene Expression Pattern in RAW Macrophage Infected with TTSS1 and TTSS1 Deletion Mutants**—The transcription factor NF-κB is involved in dozens of signaling pathways regulating many aspect of cellular activities such as cell growth and differentiation, apoptosis, stress, immune response, inflammation, and adhesion. To determine the effect of VP1686-induced NF-κB suppression on NF-κB-mediated gene expression, we infected macrophages with the mutant strains containing or lacking TTSS1 (at m.o.i. 2 for 2 h) and employed microarray technology. Using this technology, the expression pattern of more than 34,000 genes was analyzed and compared with un-infected control. Data analysis was focused on genes that were altered with more than a 2-fold change. There were about 235 genes that were differentially expressed due to the inactivity of NF-κB which are presented as a hierarchical clustering in Fig. 6 for the genes which appeared in 7 different clusters. These genes belong to different biological processes, such as apoptosis, cell death, response to stress, and transcriptional

**FIGURE 5.** VP1686 binds NF-κB in yeast and suppresses NF-κB activation in macrophages. A, plasmid expressing full-length VP1680 and the C terminus (246–388 amino acids) and N terminus of VP1686 (1–287 amino acids) fused with GAL4 DNA binding domain (BD) of pGBK7 or empty pGBK7 was co-transfected with a plasmid expressing NF-κB fused with GAL4 transactivation domain (AD) of pACT2 or empty pACT2. Interactions were detected by the growth ability on medium lacking leucine, tryptophan, and histidine (−LWH). Growth of cells lacking leucine and tryptophan (−LW) is indicative of the efficiency of the transfection. B, RAW cells were left untreated or stimulated with LPS, TTSS1-negative, TTSS2-negative, VP1686-negative, VP1686-complemented, and VP1680-negative strains of *V. parahaemolyticus*. Nuclear extracts after 2 h of infection were subjected to electrophoretic mobility shift assay (EMSA) using the NF-κB binding site of the tumor necrosis factor α promoter as a probe. The specificities of the shifted bands were determined by adding abs to p65 (arrow). Con, control.

**FIGURE 6.** Effect of TTSS1 on gene transcription in RAW cells. RAW cells either uninfected or infected with TTSS1-deleted and TTSS2-deleted strains of *V. parahaemolyticus* at an m.o.i. of 2 for 2 h. Biotin-labeled cRNA prepared from cells was hybridized, and gene expression data were obtained using Affymetrix mouse genome 430 2.0 Gene Chip. Cluster diagram 315 differentially expressed probes with -fold changes of more than 2.0 in one experimental conditions. Row, a single experiment condition; column, a single gene. Expression levels are shown in red for up-regulation and blue for down-regulation.

Fig. 5A showed that NF-κB interacted with the full-length VP1686 as well as with the N terminus of VP1686 but not with the VP1680 nor with the C terminus of VP1686. The result indicates that the well conserved Fic domain in VP1686 is not necessary for this interaction.
regulation. Expression profile of selected genes and specific pathways in which the genes are involved were classified by gene ontology (Table 2 and 3).

**DISCUSSION**

We have identified the secreted protein VP1686 as the effector molecule responsible for the induction of apoptosis in macrophages infected by *V. parahaemolyticus* and that the effect depends on TTSS1. VP1686 belongs to the Fic protein family. This family contains a central conserved motif in most diverse members including *Caenorhabditis elegans* and *Drosophila melanogaster* and suggested to be involved in the regulatory mechanism of cell division. To date, the exact molecular function of these proteins is unknown. However, we have first identified VP1686 as an inducer of apoptosis in macrophages. A previous report from our group has shown that protein VP1680 of *V. parahaemolyticus*, also secreted via TTSS1, is associated with HeLa cell toxicity (6). In this study we confirmed that the deletion mutant of VP1686 but not VP1680 fails to elicit apoptosis in macrophages. Therefore, VP1686 action may be specific to macrophages.

Macrophages are essential components of the innate immune system, vital for recognition and elimination of microbial pathogens. Macrophages use TLRs to identify common pattern of pathogens, such as LPS, and in turn activate intracellular signaling pathways related to inflammation, immunity, and pro-apoptosis as well as anti-apoptosis (21, 22). Numerous studies indicate that TLR- and MyD88-mediated signaling play an essential role in the initiation of apoptosis in bacteria-faced macrophages. Several reports showed that macrophage apo-

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**TABLE 2**

Signaling pathways modulated in macrophages infected with TTSS1 mutants

| Gene name | GenBank accession no. | ΔTT1/Con | ΔTT2/Con | ΔTT2/ΔTT1 |
|-----------|-----------------------|---------|---------|-----------|
| Rgs1      | NM_015811             | 2.54    | 33.75   | 13.3      |
| Gm3       | NM_010098             | 7.38    | 10.46   | 1.4       |
| Kif6      | NM_011803             | 1.47    | 4.19    | 2.9       |
| Csnk2a1   | BB283759              | 2.45    | 4.13    | 1.7       |
| Rgs2      | NM_010581             | 0.58    | 3.22    | 5.5       |
| Ccr7      | AV231648              | 3.05    | 2.5    | 0.8       |
| Ccr1      | AV231648              | 2.50    | 19.70   | 7.9       |
| Entpd1    | BI151440              | 14.50   | 6.75    | 0.5       |
| Ptg1      | D17406                | 0.23    | 0.27    | 1.2       |
| Jag2      | AV26681               | 0.42    | 0.21    | 0.5       |
| Gria3     | BM220576              | 5.54    | 6.91    | 1.2       |
| Akt1s1    | BF019839              | 3.72    | 4.40    | 1.2       |
| Ltbp3     | BB324823              | 0.19    | 0.10    | 0.5       |

**TABLE 3**

Functional categorization of differentially expressed genes in macrophages infected with TTSS1 mutants

| Gene name | GenBank accession no. | ΔTT1/con | ΔTT2/con | ΔTT2/ΔTT1 |
|-----------|-----------------------|---------|---------|-----------|
| Apoptosis |                       |         |         |           |
| Bnip3l    | AK018668              | 1.3     | 3.2     | 2.4       |
| Pycard    | BG084230              | 0.56    | 0.26    | 0.46      |
| Bcl2a     | LI16462               | 6.13    | 5.35    | 0.87      |
| Pdcd2     | BI526195              | 3.15    | 4.06    | 1.29      |
| Casp3     | D86352                | 11.85   | 19.46   | 1.64      |
| Bfar      | AK013874              | 2.19    | 3.93    | 1.80      |
| Dapk1     | BC026671              | 0.65    | 0.27    | 0.41      |
| Dna1a3    | AK007852              | 0.37    | 0.27    | 0.74      |
| Als2cr2   | BB277912              | 0.30    | 0.59    | 2.01      |
| Igf1      | BG092677              | 1.60    | 1.85    | 1.15      |
| Irak3     | AV228493              | 0.51    | 0.32    | 0.63      |
| Mc11      | AV318494              | 4.87    | 25.92   | 5.32      |
| Tnfrsf1b  | M60499                | 6.23    | 6.24    | 1.00      |
| Bllb4     | NM_080641             | 0.60    | 0.19    | 0.32      |
| Cell growth and differentiation | | | | |
| Esm1      | U25633                | 1.03    | 3.93    | 3.84      |
| Ifrd1     | NM_013562             | 2.86    | 4.26    | 1.49      |
| Egr1      | NM_007913             | 2.32    | 4.16    | 1.79      |
| Ccr1      | AV231648              | 2.50    | 19.70   | 7.88      |
| Snap1     | BC006946              | 1.53    | 4.73    | 3.09      |
| Rcl1      | BG071028              | 0.30    | 0.60    | 2.01      |
| Regulation of transcription | | | | |
| Cdk11c2c  | BC027026              | 0.38    | 0.19    | 0.50      |
| Rarb      | BC019432              | 0.28    | 1.09    | 3.84      |
| Rnf12     | NM_013916             | 1.50    | 3.59    | 2.39      |
| Kif6      | BC026413              | 0.72    | 0.42    | 9.00      |
| Nfkbiz    | AB026551              | 23.75   | 23.55   | 0.99      |
| Bcl3      | NM_033601             | 3.28    | 5.14    | 1.57      |
| Irf2      | NM_008391             | 0.48    | 0.21    | 0.44      |
| Ddx5      | NM_007840             | 1.63    | 3.53    | 2.17      |
| Runx3     | NM_019733             | 55.43   | 76.33   | 1.38      |
| Hmgb1     | AL648759              | 1.10    | 4.19    | 3.81      |
| Arrt      | AV237393              | 0.70    | 0.25    | 0.36      |
| Ed2       | BV296703              | 0.30    | 0.75    | 2.53      |
VP1686 Induces Macrophage Apoptosis through NF-κB Inhibition

In earlier reports using different infection models there was increasing evidence that NF-κB activation is important for self-defense and survival of macrophages when encountered with bacteria (16, 21). The NF-κB system also plays a central role in innate immunity that systematically detects and eliminates microbial pathogens by TLR-mediated gene expression. Therefore, it is conceivable that to establish pathogenic action in such a hostile environment bacteria require the delivery of a unique virulent mechanism(s). Triggering the activation of proapoptotic signals, hindering their cytotoxic effects by the antiapoptotic property of the host, such as mediated by the NF-κB, A in a series of studies, suggests that Yersinia type III secretion machinery injects YopP/YopJ protein into the macrophage, where it binds and inhibits the NF-κB-activating inhibitory κB kinase, leading to down-regulation of NF-κB activation (17, 22). DNA binding of NF-κB was also actively suppressed in apoptotic macrophages by viable E. coli-secreted and -translocated effector protein B (Esp-B) (24). Our data are consistent with the notion that translocated Vibrio effector protein VP1686 shares common property of Yersinia, Escherichia, or Shigella to mediate suppression of both basal and signal induced NF-κB activity through interference with NF-κB signaling. Unlike Yersinia or Escherichia, prior activation of macrophages by LPS was not required for VP1686-induced apoptosis. Gene expression studies have revealed differential expression of more than 235 genes between TTSS1-containing or TTSS1-lacking V. parahaemolyticus infection of macrophages. Genes that are up-regulated or down-regulated after infection include genes that participate in diverse biological processes, such as cell death, cell adhesion, signal transduction, response to stress, cell growth and/or maintenance, and transcriptional regulation (Tables 2 and 3). More importantly, about 15 different signaling pathways and 29 genes involved in apoptotic and cell growth pathways were altered in their expression profiles.

In summary, this study provides new insights into the mechanism by which V. parahaemolyticus triggers apoptosis in macrophages. By injection of VP1686, Vibrio affects the signaling networks of a highly conserved host defense system mediated by NF-κB. Although TLR represents a key inducer of NF-κB pathway, cytosolic action of VP1686 in macrophages may disrupt NF-κB activities directly without the signaling dependence from TLRs. Further investigation is required to understand the role of specific NF-κB target molecule(s) in the apoptotic pathway induced by V. parahaemolyticus.

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