J. Microbiol. Biotechnol. (2008), **18**(1), 171–175

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Translocation of VP1686 Upregulates RhoB and Accelerates Phagocytic Activity of Macrophage Through Actin Remodeling

Bhattacharjee, Rabindra N.[1], Kwon-sam Park[2], Xiuhao Chen[3], Tetsuya Iida[3], Takeshi Honda[3], Osamu Takeuchi[1,4], and Shizuo Akira[1,4]*

[1]*Akira Innate Immunity Project, Exploratory Research for Advance Technology, Japan Science and Technology Agency, Osaka University, Osaka 565-0871, Japan*
[2]*Food Science and Biotechnology Major, College of Ocean Science and Technology, Kunsan National University, Jeollabuk-do 573-701, Korea*
[3]*Department of Bacterial Infections,* [4]*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*

Received: March 27, 2007 / Accepted: June 6, 2007

Here, we report that *Vibrio parahaemolyticus* induces a rapid remodeling of macrophage actin and activates RhoB GTPase. Mutational analysis revealed that the effects depend on type III secretion system 1 regulated translocation of a *V. parahaemolyticus* effector protein, VP1686, into the macrophages. Remodeling of actin is shown to be necessary for increased bacterial uptake followed by initiation of apoptosis in macrophages. This provides evidence for functional association of the VP1686 in triggering an eat-me-and-die signal to the host.

Keywords: *Vibrio parahaemolyticus*, type III secretion system, actin remodeling, RhoB, phagocytosis

The invasion strategies used by pathogens involve a diverse array of host mechanisms including inhibition of the innate immune system and induction of apoptosis in macrophages to ensure their survival. When encountered with bacterial pathogens, multicultural organisms have also evolved sophisticated defense strategies including phagocytosis that allows phagocytes to take up pathogens and large particles such as apoptotic cells, and opsonized antigens [16]. Dynamic rearrangements of the actin cytoskeleton are central to the morphological changes in the phagocytes during apoptosis and phagocytosis [6]. Recent studies have highlighted the importance of Rho GTPase signaling pathways to these changes in cellular architecture. Rho proteins and other small GTPases are also the targets of several pathogens to induce their entry in host cells such as *Chlamydia* GTPase ADP-ribosylation factor 6 (ARF6) [4]. ARF6 activation was responsible for extensive actin remodeling necessary for bacterial uptake [2]. A broad range of bacterial pathogens, including Gram-negative bacterium *V. parahaemolyticus,* uses a type III secretion system (TTSS) to deliver effector proteins into the host cell cytosol [3, 11, 13]. The TTSS machinery is a needle-like structural complex formed by about 20 proteins, and its components are highly conserved among the bacterial species [7]. Upon translocation of the effectors into the target host cell, these molecules affect a wide variety of cellular functions, which include apoptosis, disruption of signaling networks, and damaging of the cytoskeleton [8]. A recent report demonstrates that actin polymerization induced by *Chlamydia* is preceded by the translocation of a type III regulated bacterial protein, which tranduces the signal by the pathogens to trigger its own internalization [5]. Our previous study on the DNA fragmentation pattern in RAW cells revealed that the induction of apoptosis by *V. parahaemolyticus* in these cells requires functional TTSS1 [3]. To understand the role of TTSS in bacterial pathogenesis and in immunity, we used a series of mutant strains of human-pathogenic *V. parahaemolyticus* carrying deleted genes that encode TTSS1 and 2 as well as individual secretion proteins.

In this study, we show that the TTSS1-mediated secretion of VP1686 by *V. parahaemolyticus* upregulates small GTPase protein RhoB, induces a rapid reorganization of the actin cytoskeleton into a cortical ring-like structure in both cultured and thioglycollate-elicited peritoneal macrophages, and plays a critical role in bacterial uptake.

*Corresponding author
Phone: 81-6-6879-8303; Fax: 81-6-6879-8305;
E-mail: sakira@biken.osaka-u.ac.jp

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The bacteria were cultured at 37°C with shaking in Luria-Bertani

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
E. coli		
DH5α	*hsdR recA lacZYA Φ80 lacZ*ΔM15	Novagen
SM10*λpir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu*λpir* R6K	[13]
V. parahaemolyticus		
RIMD2210633	Clinical isolate; *tdhA*$^+$, *tdhS*$^+$	[12]
POR-1	Both *tdh*(*A*,*S*) deleted from RIMD2210633	[12]
ΔTTSS-1	*ΔvcrD-1* deleted from POR-1	[12]
ΔTTSS-2	*ΔvcrD-2* deleted from POR-1	[12]
ΔVP1680	*ΔVP1680* deleted from POR-1	[11]
ΔVP1683	*ΔVP1683* deleted from POR-1	This study
ΔVP1686	*ΔVP1686* deleted from POR-1	[11]
VP1686 complemented	POR-1*ΔVP1686+VP1686*	[3]
POR-101	POR-1 containing *VP1686N-cyaA* plasmid	[12]
POR-102	POR-1 containing *cyaA* plasmid	[12]
ΔTTSS-101	ΔTTSS-1 containing *VP1686N-cyaA* plasmid	[12]
TH3996	Clinical isolate; *trh*$^+$, *ure*$^+$	[14]
Plasmid		
pT7Blue T-vector	Multicopy (ColE1 *ori*) TA cloning vector, Ap^r	Novagen
pYAK1	Suicide vector, R6K *ori*, *sacB*, Cm^r	[12]
pQBI63 GFP vector	GFP expression vector, Ap^r	Wako Ltd.

(LB) medium (for *E. coli*) and LB medium supplemented with 3% NaCl (for *V. parahaemolyticus*). Thiosulfate-citrate-bile-sucrose agar (Merck) was used to screen mutant strains. Antibiotics were used at the following concentration: ampicillin, 100 μg/ml; and chloramphenicol, 5 μg/ml.

Cell Lines

RAW264.7 cells were grown as a monolayer in DMEM (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 10 U/ml penicillin G. Mouse peritoneal macrophages were collected by peritoneal lavage with Hank's 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% FBS, 100 μg/ml streptomycin, and 10 U/ml penicillin G.

Immunoblotting

Cells, infected with or without *V. parahaemolyticus*, were washed with cold PBS and pelleted. The cells were lysed in Buffer X+BSA (100 mM Tris-HCl, pH 8.5, 250 mM NaCl, 1% [v/v] NP-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.

Construction of GFP-producing *V. parahaemolyticus*

The T7 promoter of the pQBI63 GFP vector (Wako Ltd.) was unable to express GFP in *V. parahaemolyticus* strains. This vector was digested with XbaI and BglII and its T7 promoter was replaced by the TRH promoter of *V. parahaemolyticus*. The TRH promoter region from the *V. parahaemolyticus* TH3996 strain [14] was amplified by PCR using the oligonucleotide primers 5'-AGATCTACTTTAGATTGAATA-3' and 5'-TCTAGAGTTTTAGTTTCATAA-3'. The TRH promoter region and gene for GFP protein was then obtained by digestion of pQBI63 (GFP vector) with BamHI and BglII. The fragment was ligated into the BamHI site of the pSA19CP-MCS vector [10, 14]. The GFP expression plasmid was introduced into *V. parahaemolyticus* RIMD2210633 by electroporation (1.5 kV; 1,000 Ω; 25 μF).

Construction of VP1683 Deletion Mutant

The VP1683 gene deletion mutant was constructed as described previously [13] using the following primers: VP1683-1, 5'-GGATCCTATCAATACGTCACA-3'; VP1683-2, 5'-GTAATGTACTTAGCTGGCCAATGCGAACTT-3'; VP1683-3, 5'-AAGTTCGCATTGGCCAGCTAAGTACATTAC-3'; and VP1683-4, 5'-CTGCAGGTGGGAACATGTTCG-3'.

Translocation (Adenylate Cyclase Reporter Gene) Assays

RAW 264.7 cells (2×10^5 cells) were infected with *V. parahaemolyticus* harboring the secreted-protein gene-*cyaA* fusion gene at a multiplicity of infection of 2. After 3 h of infection, the cells were washed five times gently with PBS. The cyclic AMP (cAMP) level in RAW 264.7 cells was assayed using a commercial enzyme immunoassay system, cAMP Biotrak EIA (Amersham Biosciences). The cAMP levels were expressed as numbers of picomoles per cell, because the numbers of cells were almost the same in every well in this short-time infection condition.

Fluorescence Microscopy

RAW or peritoneal (PEC) macrophages were infected with a TTSS1-containing but TTSS2-deleted mutant of GFP-producing *V. parahaemolyticus* for 30 min at the multiplicity of infection (MOI) 2. Cells were extensively washed at 30 min post-infection by PBS, fixed with 4% paraformaldehyde in PBS solution, treated with 0.1% Triton X-100 for 5 min, and stained with rhodamine-phalloidin at room temperature for 30 min. Micrographs were acquired by using a fluorescence microscope (BX-50; Olympus) equipped with a cooled charge-coupled device camera. All images were processed in exactly the same manner by using Adobe Photoshop version 5.5 software.

Phagocytosis of *V. parahaemolyticus* was observed by green fluorescence for GFP.

Phagocytosis Assay (Triton X-100 Lysis Method)

PEC cells were infected with the mutant strains of both positive and negative for VP1686, and macrophage invasion or phagocytosis assays (Triton lysis method) were done. Extracellular bacteria were killed by adding prewarmed DMEM containing 100 μg/ml of gentamicin for 90 min; macrophages were then lysed at room temperature for 5 min by 1% Triton X-100, internalized bacteria were cultured in LB medium containing 3% NaCl for overnight, and total CFU (colony forming units) were recovered and counted. The results are the mean of three independent experiments.

RESULTS

One of the characteristic effects of TTSS1 is the induction of morphological change in macrophages with a cortical ring-like structure reminiscent of the early stage of apoptosis-related cytoskeleton rearrangements. We performed F-actin staining of macrophages with rhodamine-phalloidin (Molecular Probes) upon infection with mutant *V. parahaemolyticus* strains carrying deleted genes that encode TTSS1 and 2 as well as individual secretion proteins, to determine whether any of them play any role in initiating actin remodeling in macrophages. Primarily, mutant strains lacking TTSS1, TTSS2, as well as two effector proteins (VP1686, VP1683) regulated by TTSS1 were exposed to RAW macrophages at the multiplicity of infection (MOI) 2. Staining of macrophage actin by rhodamine-phalloidin showed cell rounding, contraction, and reorganization of actin into a cortical ring-like structure, and these typical changes were only found when macrophages were infected with the mutant strains in which both TTSS1 and VP1686 were retained but TTSS2 or VP1683 were deleted. In an attempt to reveal whether *Vibrio*-induced actin reengagement is specific to VP1686 or a more general phenomenon in bacterial infection, we complemented the gene for VP1686 in a VP1686-deleted mutant strain. Consistent with VP1686-carrying wild-type strains, VP1686-complemented strains showed extensive rearrangement of macrophage actin. These results suggest that the remaining secretory components VP1686 in those strains and its delivery machinery TTSS1 in the parental strain might be the principal inducer of actin remodeling (Fig. 1A).

Previously, by using cDNA microarray analysis, we have shown a significant activation of the G-protein coupled receptor signaling pathway and its molecules such as RhoB and Rgs1 in macrophages infected with TTSS1-carrying mutant strains of *V. parahaemolyticus*. To further investigate the possible mechanism of this change, we performed Western blot analysis of macrophages infected with mutant *V. parahaemolyticus* strain carrying deleted genes that encode TTSS1 and 2 as well as individual secretion

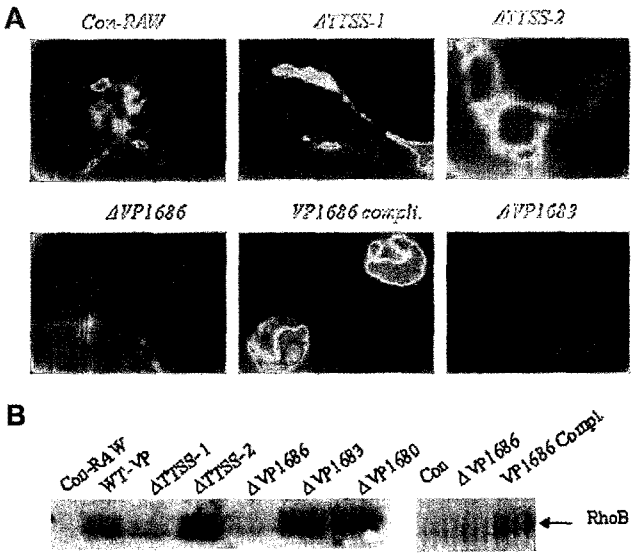

Fig. 1. TTSS1 and VP1686 are involved in macrophage actin remodeling.

A. RAW 264.7 cells were infected with mutant strains of *Vibrio parahaemolyticus* as depicted in the figures and described in the text. Actin was stained with rhodamine-phalloidin (red; all panels). **B.** RhoB is upregulated in actin remodeled macrophages. Whole-cell RAW lysates were subjected to Western blot analysis using RhoB-specific polyclonal antibodies (Santa Cruz).

proteins. Consistent with the mRNA induction, the Western blot analysis also revealed that the translocation of VP1686 is required to upregulate RhoB in these macrophages, in which typical ring-like structural changes occurred. Cells challenged by the ΔVP1686 or ΔTTSS-1 mutant strain displayed typical RAW cells. However, the ΔTTSS-2 or ΔVP1683 or VP1686-complemented strain showed rounding with shrunken cytoplasm and nuclei (Fig. 1A). To further investigate the activation of RhoB GTPase into RAW cells by translocation of VP1686, we performed Western blot analysis using anti-RhoB antibodies. The ΔTTSS-1 or ΔVP1686 deletion mutants were not detected to activate RhoB GTPase, but ΔTTSS-2, Δ1683, Δ1680, or ΔVP1686 plus VP1686 complemented mutant strains showed the increased expression of RhoB GTPase (Fig. 1B). These results suggest that the translocation of VP1686 into the RAW cells induces a rapid remodeling of RAW cells actin and activates RhoB GTPase. Moreover, in the assays for intracellular cAMP concentrations in infected RAW 264.7 cells, a large increase in intracellular cAMP levels was detected for the POR-1 strain harboring pVP1686N-*cyaA*, whereas the TTSS-1 mutant harboring the fusion gene was not (Table 2). These results suggest that the VP1686 protein was translocated into RAW cells *via* the TTSS1 apparatus, consistent with our previous result [11].

A recent study has revealed the importance of small GTP-binding protein ARF6 in regulating the structure of actin cytoskeleton, which is required for the efficient *Chlamydia* uptake [5]. To further assess the role of VP1686

Table 2. Intracellular cAMP concentrations in infected RAW 264.7 cells.

Strain	cAMP (pmol/well)
POR-1/VP1686N-CyaA	110.2
TTSS-1/VP1686N-CyaA	7.3
POR-1 carrying *cyaA*	6.9
POR-1	0.2
No strain (PBS)	0.1

in *V. parahaemolyticus* infection and host immunity, we constructed a GFP expression vector that is suitable for the expression of GFP into *V. parahaemolyticus*.

Primary mouse peritoneal macrophages (PEC) from both wild type and mouse deficient for functional Toll-like receptor (TLR) adapter MyD88 (myeloid differentiation primary response protein 88) were collected. These cells

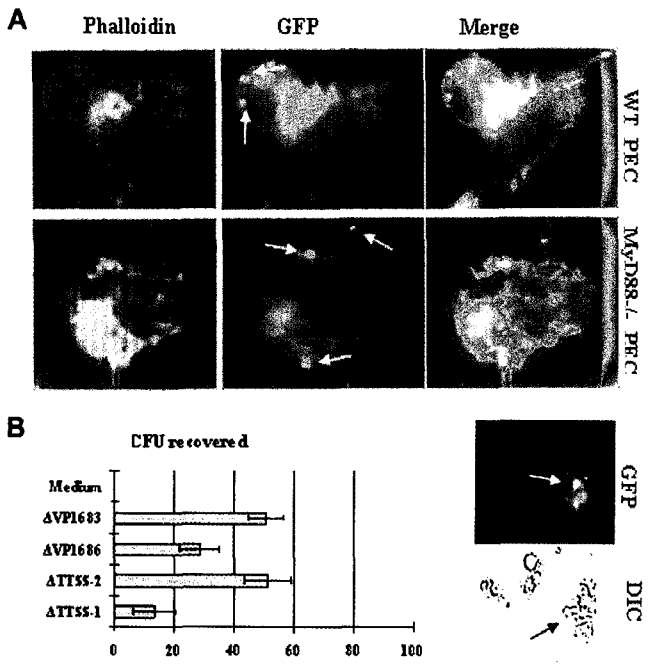

Fig. 2. Structural arrangement of actin enhances bacterial entry into PEC.

A. Peritoneal macrophages (PEC) elicited from the indicated mice were infected with GFP-producing TTSS1-positive (TTSS2-negative) mutant strains of *Vibrio parahaemolyticus*. Cells were then washed with fresh medium containing gentamicin (100 μg/ml). Actin was stained with rhodamine-phalloidin (red; left panels). GFP-expressing bacteria (indicated by arrows) were visualized as green fluorescence (middle panels) and merged images are shown in right panels. Engulfed bacteria appear green. **B.** Bacterial entry is impaired when infected by TTSS1-VP1686 deleted strains. The efficiency of bacterial entry is expressed relative to that of uninfected cells. Phagocytic ability of PEC macrophages was calculated by macrophage invasion assays (Triton X-100 lysis method), as indicated in the text, upon infection of various mutant strains of *Vibrio parahaemolyticus*, as mentioned in Fig. 2A. The results are the means of three independent experiments. **C.** Arrows show bacterial entry as green GFP fluorescence (top), and the overall structure of infected macrophages upon prolonged infection (apoptotic) can be visualized in the differential interference contrast (DIC) image (bottom).

were infected with a TTSS1-containing but TTSS2-deleted mutant of GFP-producing *V. parahaemolyticus*. Consistent with cultured macrophages in Fig. 1A, confocal analysis showed that the VP1686-containing strain of *V. parahaemolyticus* effectively induced peripheral actin remodeling in PECs. Interestingly, the observation made by green fluorescence for GFP confirms the bacterial uptake only by macrophages were changes to a cortical ring-like structural formation, and the event is independent to their TLR signaling status, which plays an active role in infection (Fig. 2A).

To further investigate whether VP1686 is implicated in the bacterial entry, PEC cells were infected with the mutant strains both positive and negative for VP1686 (as in Fig. 2B) and macrophage invasion or phagocytosis assays (Triton lysis method) were done. The results of three independent experiments (Fig. 2B) showed a considerable increase in bacterial entry when macrophages were infected with the mutant strains lacking TTSS2 or VP1683 but TTSS1 and VP1686 were retained (52 CFU and 50 CFU, respectively), compared with both TTSS1-deleted and VP1686-deleted strains (17 CFU and 24 CFU, respectively). Bacterial internalization caused membrane blebbing and cellular disintegration in macrophages upon 3–4 h post-infection (Fig. 2C), and previously we have confirmed that DNA fragmentation occurred in these macrophage cells [3].

Discussion

In this report, we have identified an effector protein of *V. parahaemolyticus*, called VP1686, as an inducer of actin remodeling in macrophages at an early infection step reminiscent of phagocytosis. VP1686 contains a central conserved motif "HPFXXGNG" in most of its diverse members and belongs to the family of proteins called Fic (filamentation induced by cAMP) suggested to be involved in the regulatory mechanism of cell division, although its exact molecular function is still unknown. Translocation of VP1686 into macrophages was directly associated with a sharp activation of the endogenous small GTP-binding protein, RhoB. Recent research has emphasized the importance of signaling pathways controlled by Rho family GTPases in regulating actin cytoskeleton structure and a target of several pathogens to induce their entry in host cells [9]. We also observed that a specific cortical ring-like actin structure was associated with phagocytic efficiency of both cultured and primary peritoneal macrophages. Furthermore, macrophages infected with both VP1686-negative and TTSS1-negative mutants of *V. parahaemolyticus* were resistant to RhoB-induced peripheral actin remodeling to a loop-like structure. The number of bacteria engulfed by these macrophages was significantly lower than the infection with TTSS1 or VP1686-containing mutants (60% and 50% inhibition, respectively). The data suggest that VP1686 plays a

significant role in these processes. In vertebrates, the main function of the innate immune system is to recognize the presence of pathogen-associated molecular patterns (PAMPs) on invading microbes and initiate downstream signals from toll-like receptors (TLRs), which lead to the expression of inflammatory response-related genes [15]. MyD88 is a common adapter shared by almost all TLRs [1, 15]. Surprisingly, macrophages lacking the toll-like receptor adaptor molecule MyD88 showed similar efficiency in bacterial uptake, indicating that actin reorganization and phagocytic activity of the host are TLR-independent. Altogether, our findings suggest that *V. parahaemolyticus* factor VP1686 stimulates the formation of a specialized structure to promote its own internalization through the activation of G-protein coupled signaling pathways including RhoB.

Acknowledgments

We thank the Akira laboratory members for technical support and Dr. Manoor P. Hande for critical reading of the manuscript. R. N. B. and K. S. P. contributed equally to this study.

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