RESEARCH LETTER

Identification and characterization of a type III secretion-associated chaperone in the type III secretion system 1 of *Vibrio parahaemolyticus*

Yukihiro Akeda[1,2], Kanna Okayama[1], Tomomi Kimura[1], Rikard Dryselius[3], Toshio Kodama[1], Kazunori Oishi[2], Tetsuya Iida[3] & Takeshi Honda[1]

[1]Department of Bacterial Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; [2]Laboratory of Clinical Research on Infectious Disease, IRCID, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; and [3]Laboratory of Genomic Research on Pathogenic Bacteria, IRCID, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Correspondence: Yukihiro Akeda, Department of Bacterial Infections, Research Institute for Microbial Disease, Osaka University, Osaka, Japan. Tel.: +81-6-6879-8276; fax: +81-6-6879-8277; e-mail: akeda@biken.osaka-u.ac.jp

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Abstract

Vibrio parahaemolyticus causes human gastroenteritis. Genomic sequencing of this organism has revealed that it has two sets of type III secretion systems, T3SS1 and T3SS2, both of which are important for its pathogenicity. However, the mechanism of protein secretion via T3SSs is unknown. A characteristic of many effectors is that they require specific chaperones for efficient delivery via T3SSs; however, no chaperone has been experimentally identified in the T3SSs of *V. parahaemolyticus*. In this study, we identified candidate T3SS1-associated chaperones from genomic sequence data and examined their roles in effector secretion/translocation and binding to their cognate substrates. From these experiments, we concluded that there is a T3S-associated chaperone, VecA, for a cytotoxic T3SS1-dependent effector, VepA. Further analysis using pulldown and secretion assays characterized the chaperone-binding domain encompassing the first 30–100 amino acids and an amino terminal secretion signal encompassing the first 5–20 amino acids on VepA. These findings will provide a strategy to clarify how the T3SS1 of *V. parahaemolyticus* secretes its specific effectors.

Introduction

The marine bacterium *Vibrio parahaemolyticus* causes acute human gastroenteritis when consumed in raw seafood (Honda & Iida, 1993). Clinical symptoms such as diarrhea have been explained by the thermostable direct hemolysin (TDH) or the TDH-related hemolysin (Honda & Iida, 1993). *Vibrio parahaemolyticus* is one of the most frequently reported bacteria isolated from food-borne infections in Japan and since 1996 it has emerged as one of the major gastroenteric pathogens found worldwide, including in southern and southeastern Asian countries, the circum-Pacific region, the Mexican Gulf and the Atlantic coasts of North and South America, and Africa (Nair *et al.*, 2007). This pattern is mostly related to specific serotypes (O3:K6, O4:K68, O1:K25 and the untypeable O1:KUT) of this bacterium, which produce the TDH responsible for the Kanagawa phenomenon (KP) (Nair *et al.*, 2007). KP is a form of hemolysis produced on a special blood agar medium called Wagatsuma agar medium, which is commonly used as an indicator for pathogenic strains of *V. parahaemolyticus* (Miyamoto *et al.*, 1969).

Whole genome sequencing of *V. parahaemolyticus* RIMD2210633, a KP-positive pandemic strain isolated from a patient who suffered traveller's diarrhea, has revealed that there are two sets of type III secretion systems: T3SS1 and T3SS2 (Makino *et al.*, 2003). The T3SS family of proteins comprises a sophisticated secretion machinery found in some gram-negative bacteria to inject virulence factors – effectors – directly into host cells (Hueck, 1998; Galan & Wolf-Watz, 2006). Several studies have shown that it is involved in the process of bacterial infection to its host (Hueck, 1998; Coburn *et al.*, 2007) and *V. parahaemolyticus* T3SSs are involved in the clinical symptoms caused by this organism (Park *et al.*, 2004). Some of the T3S-effectors in *V. parahaemolyticus* have been identified (Trosky *et al.*, 2004; Ono *et al.*, 2006; Kodama *et al.*, 2007; Liverman *et al.*, 2007; Casselli *et al.*, 2008). It has also been reported that T3SS1

and T3SS2 are responsible for cytotoxic activity against cultured cells in a *tdh*-deleted KP-positive strain of *V. parahaemolyticus* (Park *et al.*, 2004; Ono *et al.*, 2006; Kodama *et al.*, 2007).

There have been extensive recent studies of *V. parahaemolyticus* T3SSs, but how the effectors and components of the T3SS machinery are expressed and secreted is poorly understood. However, the mechanisms of effector secretion have been partly characterized in T3SSs of other bacteria, such as *Salmonella* (Patel *et al.*, 2005), *Yersinia* (Trosky *et al.*, 2008), *Shigella* (Ogawa *et al.*, 2008), *Pseudomonas* (Jin *et al.*, 2003), enteropathogenic or enterohemorrhagic *Escherichia coli* (Caron *et al.*, 2006) and other pathogenic gram-negative bacteria. Major players for this are the T3S-associated chaperones of their substrates (effectors) (Stebbins & Galan, 2003; Ghosh, 2004; Akeda & Galan, 2005). A characteristic of many bacterial effectors of T3SSs is that they require specific chaperones for efficient delivery. Cognate chaperones bind to their effectors and target the appropriate T3SS for effector secretion. One problem in studying the mechanism of protein secretion by the T3SSs of *V. parahaemolyticus* is that no chaperone has been experimentally identified, and a PSI-BLAST search previously predicted several possible T3S-associated chaperones for their cognate effectors in *V. parahaemolyticus* (Pallen *et al.*, 2005). In addition, two sets of T3SSs are able to secrete specific effectors for each T3SS independently when *V. parahaemolyticus* bacteria are grown under T3SS-inducing conditions (Fig. 1b and Y. Akeda *et al.*, unpublished data). This implies that a specific chaperone for each T3S effector might be required to recognize the appropriate T3SS for secretion. Therefore, identification of the T3S-associated chaperones is essential if we are to understand the protein delivery mechanisms of the T3SSs of *V. parahaemolyticus*. To this end, we aimed to identify the T3SS1-associated chaperone. We successfully identified and characterized the cognate chaperone, VP1682 (designated *V. parahaemolyticus* T3S-effector chaperone; VecA) for VP1680 (designated *V. parahaemolyticus* T3S-effector protein; VepA), which has been described as a T3SS1-specific effector (Ono *et al.*, 2006).

Materials and methods

Bacterial strains and growth conditions

Vibrio parahaemolyticus RIMD2210633 derivative strain POR-1 (Δ*tdhAS*) was used as the wild type in this study (Ono *et al.*, 2006). T3SS1 (Δ*vcrD1*), T3SS2 (Δ*vcrD2*) and VepA (Δ*vepA*) knockout strains of *V. parahaemolyticus* were reported previously (Park *et al.*, 2004; Ono *et al.*, 2006). All *V. parahaemolyticus* strains were grown in high-salt Luria–Bertani (LB) medium (1% Bacto tryptone, 0.5% yeast extract and 3% NaCl) at 37 °C for routine culture. For the T3SS-inducing condition, strains were grown in LB medium (1% Bacto tryptone, 0.5% yeast extract and 0.5% NaCl). *Escherichia coli* DH5α, SM10λ*pir* and BL21 (DE3) were used for general manipulation of DNA, for mobilization of the suicide vector into *V. parahaemolyticus* and for protein purification, respectively. *Escherichia coli* strains were also grown in LB medium. When necessary, media were supplemented with antibiotics: ampicillin (100 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}) and tetracycline (5 μg mL^{-1}).

Construction of *vecA*-deletion mutant strain and isogenic mutant strains encoding *vepA–cyaA* translational fusion

The Δ*vecA* strain was constructed from the Δ*tdhAS* knockout *V. parahaemolyticus* RIMD2210633 (POR-1) strain using a suicide vector, pYAK1 (R6K*ori*, *sacB*, *cat*), as reported (Kodama *et al.*, 2002; Ono *et al.*, 2006). Primer sets for deletion of *vecA* were designed as Δ*vecA*-1 (5′-GGATCCCGTAAAAATCGTCGATCC-3′), Δ*vecA*-2 (5′-TCCCTCCTACACACACGGTTCATAATCTTTCC-3′), Δ*vec A*-3 (5′-GGAAAGATTATGAACCGTGTGTGTAGGAGGGA-3′) and Δ*vecA*-4 (5′-CTGCAGGCTCACCACTTCCAAAAT-3′). For complementation, the gene *vecA* was cloned into pSA19Cm-MCS (Nomura *et al.*, 2000). Isogenic mutants of the translational fusion, $vepA_{1–105}$–$cyaA_{4–1215}$ (*cyaA* encoding adenylate cyclase from *Bordetella pertussis*) in wild type and Δ*vecA* strains were constructed by homologous recombination using pYAK1.

Secretion assay

Bacterial strains were grown under T3SS-inducing conditions at 37 °C for 18 h and subcultured in 5 mL of LB medium for 3 h. After collecting culture supernatant and bacterial pellets by centrifugation, supernatants were filtered with 0.22-μm-pore filters. Supernatants were mixed with sodium deoxycholate and ice-cold trichloric acetate at final concentrations of 0.1% and 10%, respectively. Mixed samples were kept on ice for 1 h and centrifuged to yield pellets. Pellets were washed with ice-cold acetone followed by centrifugation. Final samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before Western blotting with the appropriate antibodies.

RNA isolation and reverse transcription (RT)-PCR

Bacteria were grown under the same conditions as for the secretion assay. After incubation, bacterial cultures were applied to RNeasy Mini kits (Qiagen, Valencia, CA) following the manufacturer's protocol. Isolated RNA (2 μg) was reverse transcribed into cDNA using SUPERSCRIPT III (Invitrogen, Carlsbad, CA) and random hexamers (Takara Bio,

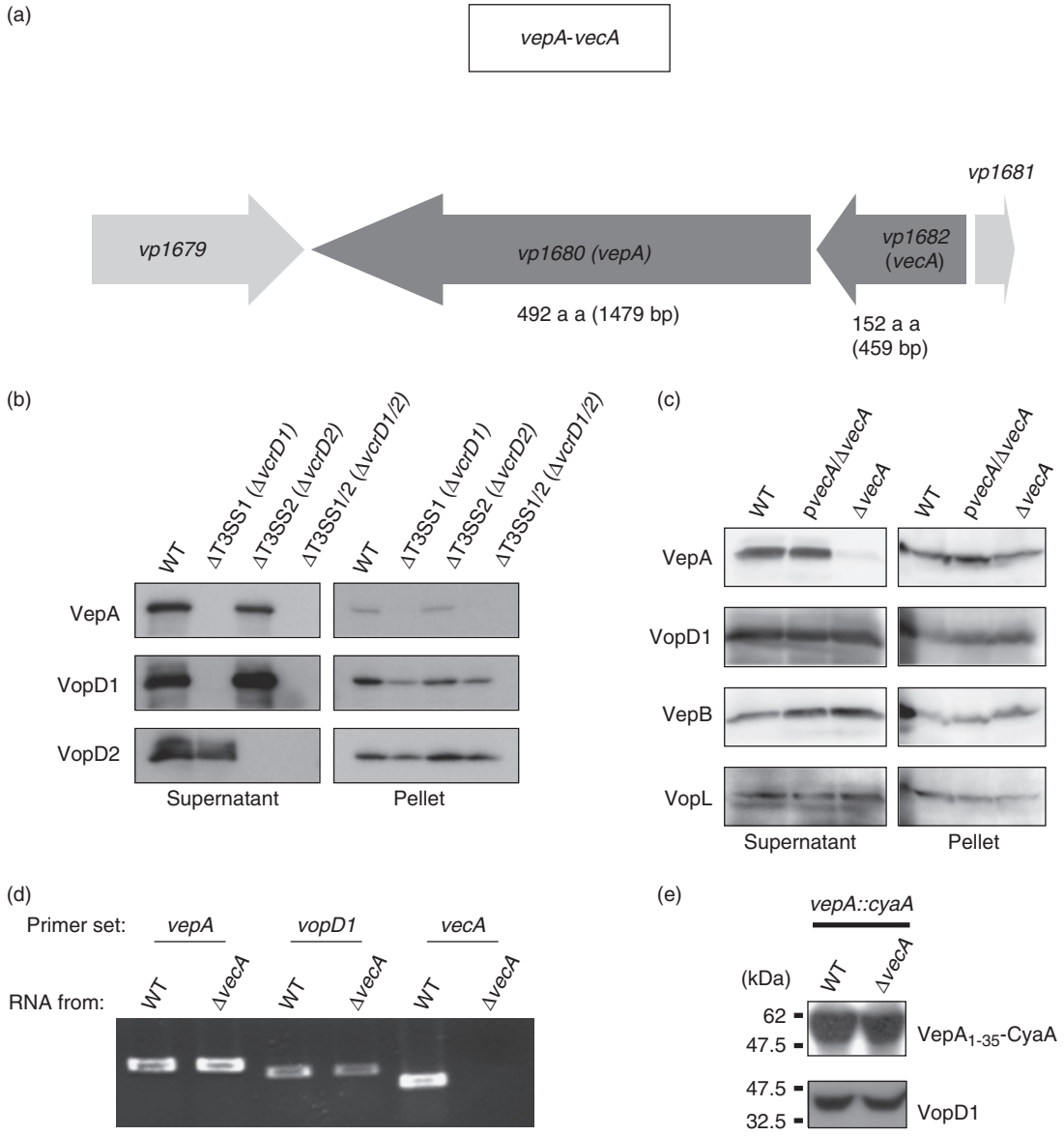

Fig. 1. VecA is required for T3SS1-specific VepA secretion. (a) The loci of *vecA* and *vepA* on *Vibrio parahaemolyticus* RIMD2210633 chromosome I. (b) Secretion assay using T3SS1- or T3SS2-deficient strains. *Vibrio parahaemolyticus* strains [wild type (WT), Δ*vcrD1*, Δ*vcrD2* and Δ*vcrD1/D2*] were grown under T3SS-inducing conditions. Culture supernatants and bacterial pellets were applied to Western blotting detected by antibodies against VepA, VopD1 and VopD2. (c) Secretion assay using the Δ*vecA* strain. *Vibrio parahaemolyticus* strains [WT, Δ*vecA* and Δ*vecA* complemented with plasmids encoding *vecA* (p*vecA*/Δ*vecA*)] were grown under T3SS-inducing conditions. Culture supernatants and bacterial pellets were used in Western blotting and detected by rabbit polyclonal antibodies against VepA, VopD1, VepB and VopL. (d) RT-PCR of *vepA* in *V. parahaemolyticus* strains. Amplification was carried out using RNA extracted from WT and Δ*vecA* strains. Primers for *vopD1* and *vecA* were used for positive and negative controls, respectively. (e) Expression of $VepA_{1–35}$–CyaA fusion in *V. parahaemolyticus* strains (WT and Δ*vecA*) carrying $vepA_{1–105}$–CyaA. Strains were grown under T3SS-inducing conditions. $VepA_{1–35}$–CyaA in bacterial pellets was detected by Western blotting with an anti-CyaA monoclonal antibody. Anti-VopD1 was used for internal control.

Shiga, Japan). The acquired cDNA was used for semi-quantitative PCR amplification of internal fragments of *vepA*, *vopD1* and *vecA* with specific primers for *vepA* (5′-CGCACTTTTGGCTTATTCATTAGTCAGTCC-3′ and 5′-TCCTGCATGCTAAAAGTGAAATCGCCCTTC-3′), *vopD1* (5′-GATCTACTGATGAGTACTACCGCGAAAGTG-3′ and 5′-CTCATCTGCTTTTTGCTTCGCTGCTTGAGC-3′) and *vecA* (5′-AGAACACTGCGAGAAATCGTGTACAAAAC-3′ and 5′-TAGTTTCACTTCAGAAGCCATTTTATTAAATAAAG-3′). PCR reactions were performed as follows; one cycle at 98 °C

for 3 min; 30 cycles at 98 °C for 10 s, 53 °C for 30 s and 72 °C for 1 min followed by one cycle at 72 °C for 7 min. PCR products were analyzed by 2% agarose gel electrophoresis.

Cytotoxicity assay

Cytotoxicity was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay kits (Promega, Madison, WI) as described (Ono *et al.*, 2006; Kodama *et al.*, 2007). In brief, Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 in air, then bacterial strains were added at a multiplicity of infection of 100. At 3 h of infection, supernatants were collected and the release of lactose dehydrogenase was quantified using the kit.

Purification of glutathione-*S*-transferase (GST)-VecA and polyhistidine-tagged VepA

VecA fused with GST was expressed by the plasmid pGEX-6P1 (GE Healthcare Bio-Sciences, Little Chalfont, UK) encoding *vecA* amplified by PCR. *Escherichia coli* BL21 (DE3) harboring the plasmid expressing GST-VecA was grown in 2 × YT medium (1.6% Bacto tryptone, 1% yeast extract and 0.5% NaCl) for 18 h, then subcultured in 2 × YT medium for 3 h at 37 °C. After the addition of isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1 mM, bacterial culture was incubated for 4 h. Harvested bacterial pellets were suspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM dithiothreitol and 0.1% Triton X-100, pH 8.0) with 10 μg mL^{-1} of lysozyme, 10 μg mL^{-1} of RNAse and 5 U of DNAse I, then centrifuged at 20 000 ***g*** for 20 min. Cleared lysate was loaded onto a glutathione Sepharose 4 Fast Flow column (GE Healthcare Bio-Sciences) equilibrated with lysis buffer. After washing the column with washing buffer (20 mM Tris-HCl, 500 mM NaCl and 2 mM dithiothreitol, pH 8.0), GST-VecA was eluted with elution buffer (20 mM Tris-HCl, 200 mM NaCl and 10 mM glutathione, pH 8.0).

Polyhistidine-tagged VepA was expressed on the plasmid pET28a (Novagen, Madison, WI) encoding *vepA* amplified by PCR. Recombinant protein was expressed in *E. coli* BL21 (DE3). Purification of polyhistidine-tagged VepA was carried out using Ni-NTA His · Bind beads (Novagen) following the manufacturer's instructions.

Pulldown assay using GST-VecA and polyhistidine-tagged VepA or VepA fused with CyaA

Purified GST-VecA was absorbed onto glutathione beads equilibrated with TBST buffer (20 mM Tris-HCl, 200 mM NaCl and 0.05% Tween 20, pH 8.0). Beads were washed to remove unbound protein, then suspended in TBST containing purified polyhistidine-tagged VepA or *E. coli* lysates expressing a series of truncated VepA forms fused with CyaA. After incubation for 1 h at 4 °C, beads were washed with TBST five times. Washed beads were applied to SDS-PAGE followed by Western blotting using anti-GST antibody (Cell Signaling Technology, Danvers, MA), antipolyhistidine-tag antibody (Sigma-Aldrich, St. Louis, MO) or anti-CyaA (Santa Cruz Biotechnology, Santa Cruz, CA).

Results and discussion

Identification of a T3SS1-associated chaperone

Previous studies suggested that T3S-associated chaperones are often encoded in the vicinity of the genes for their cognate effectors; the estimated molecular weights are usually < 15–20 kDa and isoelectric points of chaperones are acidic (Feldman & Cornelis, 2003; Parsot *et al.*, 2003). Considering this background information, we attempted to predict which gene might encode for the chaperone of VepA, based on the genomic sequence of *V. parahaemolyticus* RIMD2210633 (Makino *et al.*, 2003).

One candidate chaperone gene, *vp1682* (designated *V. parahaemolyticus* T3S-effector chaperone; *vecA*), is encoded next to *vepA* and its estimated molecular weight is 17.1 kDa (Fig. 1a). This candidate was also predicted as a possible T3S-associated chaperone previously (Pallen *et al.*, 2005). Before we examined the role of VecA as a chaperone for VepA, we first tested whether VepA was secreted specifically via T3SS1 using a secretion assay of T3SS1- or/and T3SS-deficient mutant strains (Fig. 1b). The secretion assay was performed as described in Materials and methods. Proteins were detected using antibodies against VepA, VopD1 (VP1656, a T3SS1-specific translocon) and VopD2 (VPA1361, a T3SS2-specific translocon) (Kodama *et al.*, 2008). This experiment indicated that VepA was a T3SS1-specific effector, although the amounts of VepA inside T3SS1-deficient strains were only slightly expressed by a negative regulator, ExsD. In *Pseudomonas aeruginosa* T3SS it has been shown that ExsD binds to ExsC to suspend negative regulation under T3SS-inducing conditions. On the other hand, under non-T3SS-inducing conditions, ExsC binds to ExsE, which is secreted through T3SS. ExsD then represses T3SS gene transcription (Urbanowski *et al.*, 2005). There are homologues of ExsC and ExsE in the T3SS1 locus of the *V. parahaemolyticus* genome (Zhou *et al.*, 2008), so it would appear that the T3SS1-deficient strains could not secrete ExsE, resulting in the absence of expression of other T3SS1-related proteins. As shown in Fig. 1c, as mentioned above, many of the T3S-associated chaperones are required for the secretion of their effectors; so we assayed the secretion of VepA using the Δ*vecA* strain. Secreted proteins

and bacterial pellets were detected by rabbit polyclonal antibodies against VepA, VP1686 (designated *V. parahaemolyticus* T3S-effector protein; VepB), which has been also described as a T3SS1-specific effector (Ono *et al.*, 2006; Casselli *et al.*, 2008), VopD1 (Ono *et al.*, 2006) and VopL (VPA1370, a T3SS2-specific effector) (Liverman *et al.*, 2007). As shown in Fig. 1c, the secretion of VepA was detected in the wild-type strain and the complemented strain, but not in the Δ*vecA* strain. Cytoplasmic VepA was expressed equivalently in each strain except for Δ*vecA*, and this indicated that VecA is required either for the secretion and stability of VepA in bacterial cytoplasm or for its transcription. To examine whether VecA might be required for the entire T3SS function to secrete proteins, VopD1, VepB and VopL were also detected. There were no differences in the amounts of proteins targeted by antisera against VopD1, VepB and VopL among the three strains assayed.

Some T3S-associated chaperones can control the transcription of T3S-associated genes (Feldman & Cornelis, 2003; Parsot *et al.*, 2003). To test this possibility for VecA, the transcriptional level of *vepA* in the Δ*vecA* strain was also evaluated by RT-PCR. Both wild-type and Δ*vecA* strains were grown under T3SS-inducing conditions and RNA was extracted and applied to RT-PCR. Both strains were able to show a similar level of PCR products amplified with the primer sets of *vepA* and *vopD1* (Fig. 1d), indicating that the transcription of *vepA* was regulated without *vecA*, compared with the positive control of *vopD1*. The translational level of *vepA* in the Δ*vecA* strain was also evaluated using translational fusion with the first 2–405 amino acids of CyaA from *B. pertussis*. Both isogenic mutants of $vepA_{1-105}$–*cyaA* in the wild-type and Δ*vecA* strains were able to express a similar level of the translational fusion detected by anti-CyaA under the same conditions as the secretion assay (Fig. 1e). From these results, it appears that VecA does not play a role in regulating the transcription/translation of VepA and that it seems to be the chaperone necessary for the secretion of VepA through T3SS1. This result is also consistent with a previous prediction (Pallen *et al.*, 2005).

Translocation of VepA into culture cells is dependent on VecA

Translocation of T3S effectors into host cells is thought to be a different event from effector secretion into culture media (Lee & Galan, 2003; Ehrbar *et al.*, 2006). Secretion of amino-terminal peptides of effectors fused with reporter proteins into culture medium has been observed even in T3SS-deficient bacterial strains (Lee & Galan, 2004); however, translocation of effectors requires complete T3SS. To rule out the possibility that VecA might not be required for the translocation of VepA, we carried out a cytotoxicity assay. VepA is a cytotoxic effector against cultured cells (Ono *et al.*, 2006) and can be used to evaluate the translocation of VepA, which shows its cytotoxicity inside cultured host cells after injection via T3SS1. To examine the role of VecA in the translocation of VepA, *V. parahaemolyticus* strains were used in a cytotoxicity assay using the human colon carcinoma cell line, Caco-2. As shown in Fig. 2, the wild-type strain showed strong cytotoxicity against Caco-2 cells but the Δ*vecA* strain showed a similar level of cytotoxicity as the Δ*vepA* strain. This result suggested that VecA is essential not only for secretion into the culture medium but also for the translocation of VepA into host cells.

Determination of the VecA-binding site and secretion signal on VepA

Another characteristic of T3S-associated chaperones is their ability to bind to effectors (Ghosh, 2004). Therefore, we performed pulldown assays to examine the ability of VecA to bind with VepA. VecA fused with GST or GST alone was used for pulldown assays. As shown in Fig. 3, only GST–VecA, not GST alone, could bind to VepA. This binding between VecA and VepA suggests that VecA is the cognate chaperone for VepA and that it is required for the secretion and translocation of VepA.

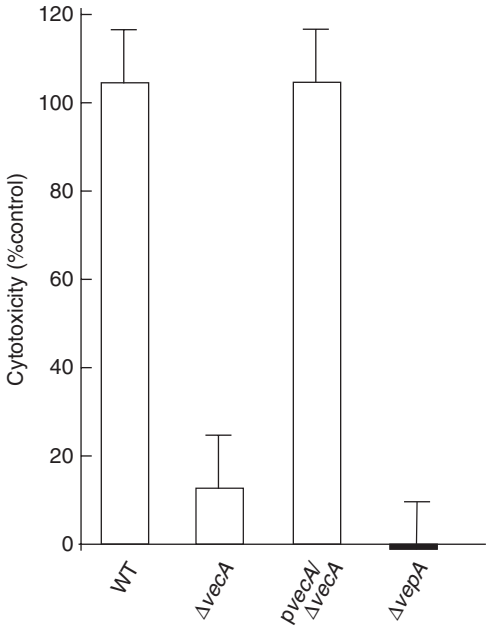

Fig. 2. Decreased cytotoxicity of Δ*vecA* strain against Caco-2 cells. Caco-2 cells were infected with wild type (WT), with Δ*vecA* and with complemented Δ*vecA* and Δ*vepA* strains of *Vibrio parahaemolyticus* in 5% CO_2 at 37 °C for 3 h. Cytotoxicity was measured by lactose dehydrogenase release from Caco-2 cells using the kit mentioned in Materials and methods. Caco-2 cells treated with lysis buffer contained in the kit were considered 100% lysed. Bars represent the means ± SD of three independent experiments.

To better understand the interaction between VepA and VecA, we next constructed a series of truncated VepA forms fused with $CyaA_{2–405}$ to determine the VecA-binding site on VepA. Following the protocol of the binding assay mentioned above, glutathione Sepharose 4B beads bound with GST–VecA were incubated with *E. coli* lysates containing a series of VepA–CyaA fusions. The result showed that the domain covering the first 30–100 amino acids of VepA was responsible for binding to GST–VecA (Fig. 4). In general, it has been demonstrated that the first *c.* 100 amino acids of T3S effectors contain the chaperone-binding domain, consistent with our results (Feldman & Cornelis, 2003; Parsot *et al.*, 2003; Ghosh, 2004; Galan & Wolf-Watz, 2006).

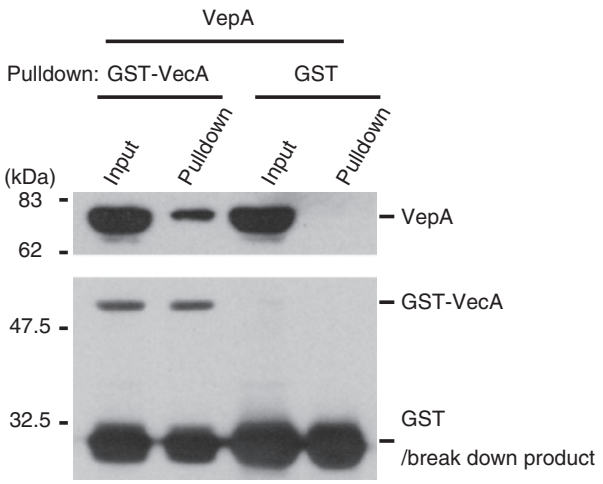

Fig. 3. Binding between VepA and VecA. Purified GST-VecA or GST alone was preabsorbed with glutathione beads and mixed with purified VepA tagged with polyhistidine. Input samples (Input) and washed beads (Pulldown) were applied to Western blotting to detect fusion proteins using anti-GST and antipolyhistidine tag antibodies.

Two regions on T3S effectors are important for their efficient secretion. One, mentioned above, is the discrete domain for binding to its cognate chaperone; the other is the amino terminal secretion signal. Although this signal is poorly conserved in terms of primary amino acid sequence among other T3S effectors, the sequence at the amino terminus is required for the efficient secretion of T3S effector proteins through T3SSs (Feldman & Cornelis, 2003; Ghosh, 2004). To test whether the amino-terminal secretion signal of T3SS exists on VepA, secretion assays using a series of truncated VepA–CyaA fusions were performed in T3SS1- or T3SS2-deficient strains (Δ*vcrD1* or Δ*vcrD2*) (Park *et al.*, 2004; Ono *et al.*, 2006; Kodama *et al.*, 2008) to observe the specific secretion of VepA–CyaA fusions by T3SS1. This assay revealed that first 5–100 amino acids were required for the secretion of the VepA–CyaA fusion product through T3SS1 (Fig. 5). However, faint signals of CyaA fusions with $VepA_{1–20}$, $VepA_{1–30}$ and $VepA_{1–35}$ were visible for the ΔT3SS1 and ΔT3SS2 strains. This indicates that the amino acid terminal secretion signal of VepA can recognize any of the T3SSs in *V. parahaemolyticus* to be secreted, although an effector with a chaperone-binding domain is likely to be restricted to one specific T3SS for efficient secretion. This means that VecA might be necessary for the specificity of VepA secretion via T3SS1. Consistent with the binding assay, the chaperone-binding domain (30–100 amino acids) of VepA was found to be required but not sufficient for its secretion. In addition, $VepA_{20–100}$–CyaA and $VepA_{1–100\ (\Delta 6–29)}$–CyaA, which have chaperone-binding domains, failed to be secreted. Taken together, these experiments indicated that the first 5–20 amino acids form the amino terminal secretion signal of VepA.

In summary, we identified VecA as a candidate for T3S-associated chaperones for VepA, based on genomic sequence

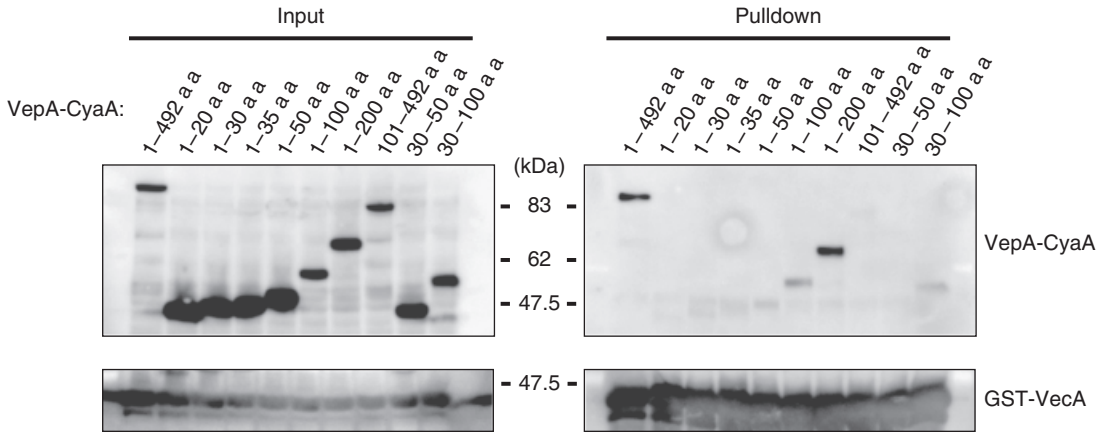

Fig. 4. Binding of truncated VepA–CyaA fusions with VecA. Whole *Escherichia coli* lysates expressing a series of truncated VepA–CyaA fusion proteins were applied to glutathione beads preabsorbed with GST–VecA. After washing the beads to remove unbound VepA–CyaA fusions, input samples (Input) and washed beads (Pulldown) were applied to SDS-PAGE separation followed by Western blotting using anti-GST and anti-CyaA antibodies.

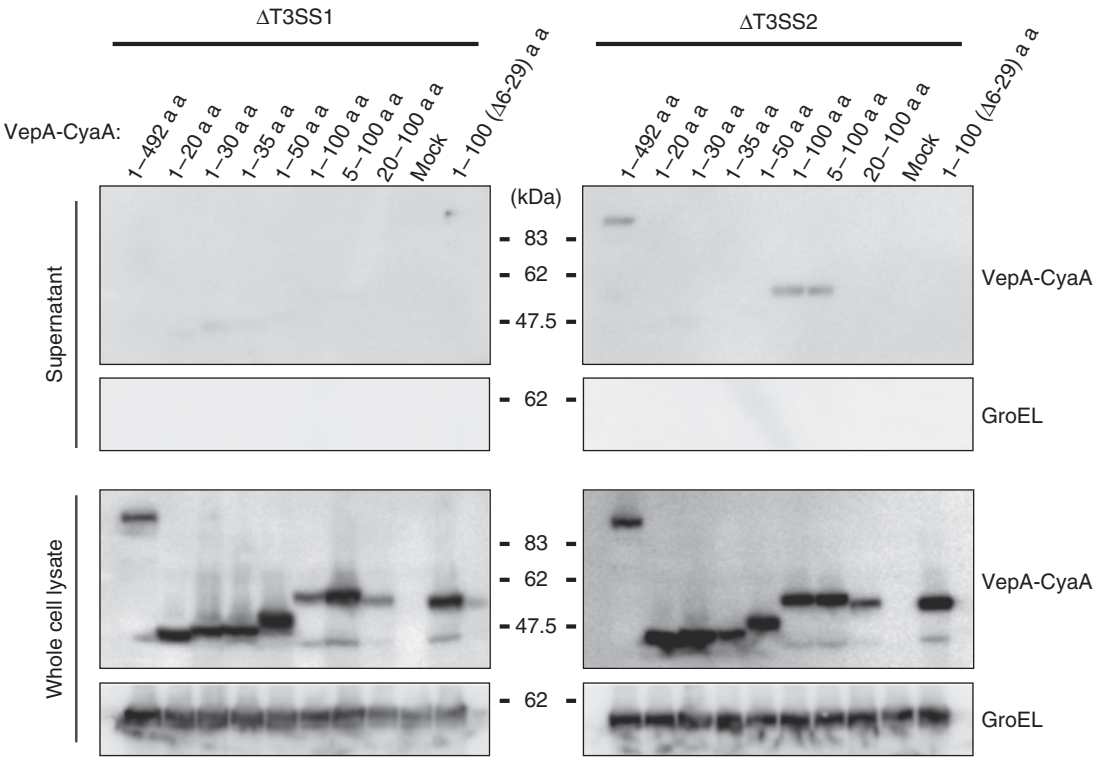

Fig. 5. Secretion of VepA–CyaA fusions. A series of truncated VepA–CyaA fusion proteins were expressed in Δ*vepA* strains with *Vibrio parahaemolyticus* T3SS1 or T3SS2 knockout backgrounds. After incubation under T3SS-inducing conditions for 3 h, culture supernatant and bacterial pellets were collected and applied to SDS-PAGE separation followed by Western blotting using anti-CyaA and anti-GroEL as a marker for the leakage of cytoplasmic proteins.

data of *V. parahaemolyticus* RIMD2210633 and examined its role for effector secretion and expression using the knockout strain and binding to their cognate substrates. This candidate is the first experimentally identified T3S-associated chaperone of T3SS1 in *V. parahaemolyticus*, and we demonstrated that the chaperone-binding site on VepA is located at the first 30–100 amino acids. Another essential locus for effector secretion, the amino terminal secretion signal, was identified at the first 5–20 amino acids. From these results, VecA seems to be a class IA chaperone, which associates with one effector, such as *Yersinia* SycE, SycH and *Salmonella* SicP (Cornelis & Van Gijsegem, 2000; Parsot *et al.*, 2003). Moreover, a BLAST search showed that VepA–VecA tandem sequences of *V. parahaemolyticus* are also found in the genome sequences of other *Vibrio* species, including *Vibrio alginolyticus* 12G01, *Vibrio harveyi* HY01 and *Vibrio* sp. Ex25 at an amino acid identity around 80%; so similar mechanisms of protein secretion and pathogenicity are likely to exist among these species.

The evidence provided here will provide a strategy to clarify how T3SS1 of *V. parahaemolyticus* secretes its effectors. The T3S effectors first interact with cognate chaperones specifically; these protein complexes are then targeted to the specific T3SS, and then T3S-associated chaperones interact with a T3S-associated ATPase and prime unfolding effectors to be ready for secretion (Akeda & Galan, 2005; Galan & Wolf-Watz, 2006). It is likely that other interactions with T3SS components also help specific targeting for effector secretion, but the effector–chaperone complex clearly plays a key role in such specificity. Importantly, our findings may reveal the determinants of effector specificity for T3SS1 and T3SS2 in *V. parahaemolyticus*.

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