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NOTE

ORF13 in the Type III secretion system gene cluster of *Edwardsiella tarda* binds to the mammalian factor Cugbp2

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ABSTRACT: The Type III secretion system (TTSS) is essential for the intracellular replication of *Edwardsiella tarda* in phagocytes of fish and mammals, and a hypothetical gene (*orf13*) located in the TTSS gene cluster is required for intracellular replication and virulence of *E. tarda*. Here, we show that under TTSS-inducing conditions, the protein ORF13 was secreted into culture supernatant. Then, using a yeast 2-hybrid screen, we show that the mammalian factor Cugbp2, which regulates apoptosis in breast cancer cells, directly interacts with ORF13. A pull-down assay revealed that ORF13 binds to the C-terminal region of Cugbp2. Our results suggest that ORF13 may facilitate *E. tarda* replication in phagocytes by binding to Cugbp2.

KEY WORDS: *Edwardsiella tarda* · Type III secretion system · ORF13 · Mammalian factor Cugbp2

INTRODUCTION

Edwardsiella tarda, a member of the family *Enterobacteriaceae*, infects a wide variety of invertebrates and vertebrates, such as fish, amphibians, reptiles, birds, and mammals, including humans. Although the mechanism of pathogenicity of *E. tarda* is still largely unknown, some virulence factors related to the pathogenicity of this organism have been reported: these include the ability to invade and replicate in epithelial cells, such as HeLa and Hep-2 (Marques et al. 1984, Janda et al. 1991, Okuda et al. 2008), and in phagocytic cells, such as fish phagocytes and murine macrophages (Srinivasa Rao et al. 2001, Okuda et al. 2006), and the ability to produce catalases, hemolysins, and dermato- or lethal toxins (Srinivasa Rao et al. 2003a, Janda & Abbott 1993, Ullah & Arai 1983, Suprapto et al. 1996). The weapon possessed by many pathogenic bacteria, the Type III secretion system (TTSS), was also identified as a very important virulence factor in *E. tarda* (Tan et al. 2002, Srinivasa Rao et al. 2003b, 2004). TTSS injects effector proteins into the cytoplasm of host cells to modify host cell physiology or to disrupt host immunity, and these proteins are important for virulence (Hueck 1998). The TTSS gene cluster consists of genes encoding effectors, regulators, apparatuses, chaperones, and translocons (Fig 1A). The *E. tarda* TTSS consists of 35 open reading frames (ORFs), and is essential for *E. tarda* survival and replication in J774 murine macrophages (Okuda et al. 2006). Okuda et al. (2014) reported that the 5 genes (*escC*, *orf13*, *orf19*, *orf29*, and *orf30*) located within the TTSS gene cluster are required both for survival and replication within J774 murine macrophages and HEp-2 human epithelial cells, and for virulence in zebrafish. In this

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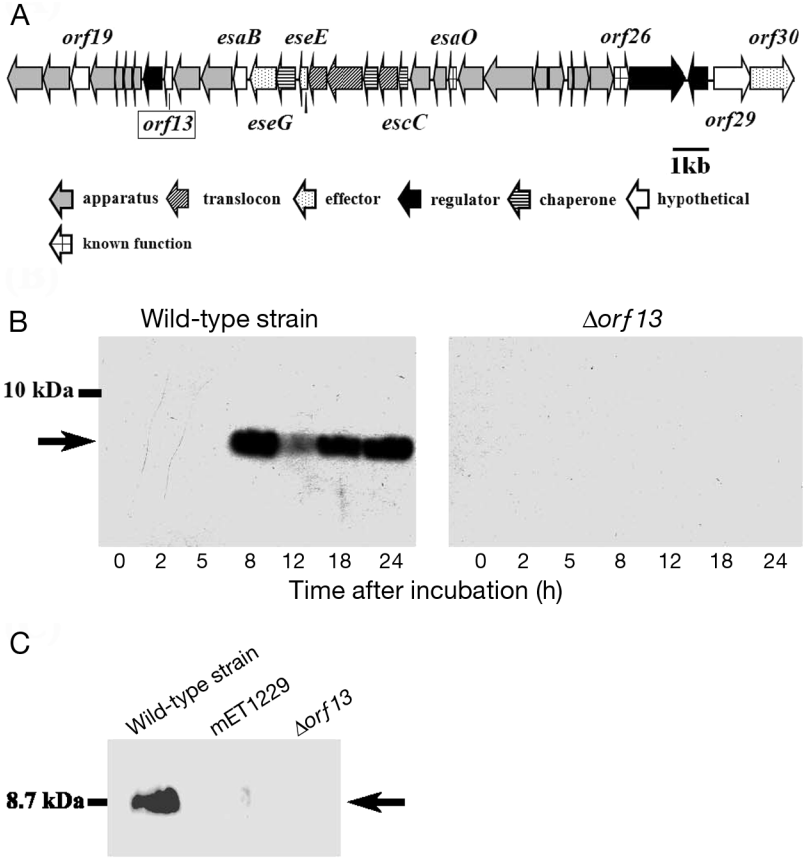

Fig. 1. (A) *Edwardsiella tarda* TTSS gene cluster, composed of 35 genes encoding apparatuses, translocons, effectors, regulators, chaperones, known function and hypothetical proteins (Okuda et al. 2014). (B) Western blot analysis to detect secretion of ORF13 into the culture supernatant of (left panel) the wild-type strain and (right panel) the Δ*orf13* mutant under TTSS-inducing conditions. The arrow shows the presence of ORF13 with a deduced molecular weight of 8.7 kDa. (C) Western blot analysis to detect secretion of ORF13 at 18 h after incubation into the culture supernatant of the wild-type strain, a TTSS-deficient mutant (mET1229), and the Δ*orf13* mutant under TTSS-inducing conditions. The arrow shows the presence of ORF13 with a deduced molecular weight of 8.7 kDa

study, we focus on the *orf13* gene in the TTSS gene cluster and show that, under TTSS-inducing conditions, ORF13 was secreted into the culture supernatant and interacted with the mammalian factor Cugbp2, which regulates apoptosis in breast cancer cells.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *Edwardsiella tarda* strain FK1051 was isolated from a diseased Japanese flounder. mET1229 is a TTSS-deficient mutant of FK1051 (Okuda et al. 2006). The *orf13* mutant of FK1051 (Δ*orf13*) was a strain with an insertion mutation in the *orf13* gene, and our laboratory stock strain is described by Okuda et al. (2014).

Identification of ORF13 in the culture supernatant under TTSS-inducing conditions

The highest expression of the EseBCD translocon proteins, secreted through the TTSS, is induced when the wild-type strain is incubated at 25 to 30°C in neutral or alkaline pH (Okuda et al. 2009). According to this observation, in a time course experiment, the wild-type strain and the *orf13* mutant were incubated for 0, 2, 5, 8, 12, 18 and 24 h at 30°C in Tripto-soy broth (TSB, Nissui) medium (pH7.3). The wild-type strain, mET1229, and the *orf13* mutant were incubated for 18 h at 30°C in TSB (pH7.3). Secreted proteins in the culture supernatant were isolated using trichloroacetic acid precipitation as described previously (Okuda et al. 2009). Western blot analysis was performed using anti-ORF13 polyclonal antibody, which was prepared by immunizing a rabbit with a synthetic oligopeptide corresponding to amino acids 61 to 74 (VRVIDTLWTLHQGTC) of ORF13.

Yeast 2-hybrid assay

Yeast 2-hybrid screening with a mouse brain cDNA fusion library (Invitrogen) was carried out as described previously (Okuda et al. 2005). The Gal4DBD-*orf13* (full-length) fusion construct (pDBLeu-*orf13*) was generated by inserting the *Msc* I–*Not* I fragment of the full-length *orf13* gene into the *Msc* I–*Not* I restriction endonuclease cleavage site within the open-reading frame of the Gal4DBD. The yeast strain MaV203 was transformed simultaneously with both pDBLeu-*orf13* and a mouse brain cDNA fusion library inserted into the activation domain vector, pPC86 (Invitrogen). Transformants were selected using a HIS3 reporter by seeding onto selective plates containing 3AT, with MaV203 harboring both

pDBLeu-*orf13* and pPC86 used as a negative control. The positive clones were confirmed using another promoter system that induces β-galactosidase expression; induction of β-galactosidase results in a blue color by seeding onto selective plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Wako). The cDNA inserts in the positive clones were sequenced, then the sequences were entered into the website http://blast.ncbi.nlm.nih.gov/ to perform the BLAST analysis.

Expression of candidate host proteins, identified by Yeast 2-hybrid assay

The full-length genes encoding the CUG triplet repeat RNA binding protein 2 (Cugbp2), secretogranin II, dynamin I, and Ena-vasodilator stimulated phosphoprotein (Ena-VASP) were PCR-amplified using a mouse brain cDNA library (Invitrogen) as the template and the following primers: Cugbp2 (5′-GAG ACT CGA GAT GCG CTG TCC CAA ATC CGC-3′ and 5′-AGA GGC GGC CGC TCA GTA AGG TTT GCT GTC GT-3′); secretogranin II (5′-GAG AGC GGC CGC ATG GCT GGA GCT AAG GCG TA-3′ and 5′-AGA GGC GGC CGC TTA CAT GTT TTC CAT GGC CC-3); dynamin I (5-GAG ACT CGA GAT GGG CAA CCG CGG CAT GGA-3′ and 5′–AGA GGC GGC CGC TCA GAG CCT GGC TTT GCC AG-3′); and Ena-VASP (5′-GAG ACT CGA GAT GAG TGA ACA GAG TAT CTG CCA A-3′ and 5′-AGA GGC GGC CGC TTA CGT GGT GCT GAT CCC ACT T-3′). The amplified fragments were cloned into an expression vector in mammalian cells, pCDL-SRα/Neo with Myc-tagged sequences, for the expression of Myc-tagged Cugbp2, Myc-tagged secretogranin II, Myc-tagged dynamin I, and Myc-tagged Ena-VASP, and the resulting plasmids were designated as pCugbp2, pSecII, pDynaI, and pEnaVASP, respectively. The 2 deleted genes, encoding Cugbp2 lacking the C-terminal 102 amino acids and Cugbp2 lacking the C-terminal 202 amino acids, were PCR-amplified using the primers (5′-GAG AGG ATC CAT GCG CTG TCC CAA ATC CGC-3′ and 5′-AGA GGC GGC CGC TCA CAG CAA GCT CTG GCT GTA CAA-3′) and the primers (5′-GAG AGG ATC CAT GCG CTG TCC CAA ATC CGC-3′ and 5′-AGA GGC GGC CGC TCA GCT AGA CAG AGG GTT TGC ATT-3′), respectively. The 2 deleted genes were cloned into pCDL-SRα/Neo with Myc-tagged sequences for expression of Myc-tagged Cugbp2ΔC102 and Myc-tagged Cugbp2ΔC202, and the resulting plasmids were designated as pCugbp2ΔC102 and pCugbp2ΔC202, respectively.

The plasmids pCugbp2, pSecII, pDynaI, pEnaVASP, pCugbp2ΔC102, and pCugbp2ΔC202 were transfected into Hep-2 cells, a carcinoma human larynx cell line, using FuGENE 6 Transfection Reagent (Roche). After 48 h of incubation in 5% CO_2-enriched atmosphere at 37°C, the Hep-2 cells were washed twice with PBS, and then lysed in 400 µl of NET-N+ buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin).

Pull-down assay to determine binding of ORF13 to candidate host proteins

The pGEX-4T-1 GST expression vector was purchased from GE Health Care. The *orf13* full-length gene was PCR-amplified using the primers (5′-GAG AGG ATC CAT GCC CAC CCT AAC GCA TCT T-3′ and 5′-AGA GGC GGC CGC TCA GGT GCC TTG GTG CAG CGT-3′) and cloned into pGEX-4T-1, and the resulting plasmid was designated as pORF13. The glutathione S-transferase fusion protein (GST-ORF13) was purified from *E. coli* DH5α (pORF13) as described previously (Toyotome et al. 2001). Briefly, *E. coli* cells carrying pORF13 were incubated in Luria-Bertani broth supplemented with ampicillin for 3 h at 37°C. Expression was induced by the addition of 1 mM isopropyl-1-thio-D-galactopyranoside and incubation for 2 h at 37°C. Bacteria were disrupted by sonication, then the GST-ORF13 fusion protein was purified using glutathione-sepharose 4B (GE Healthcare Life Sciences) according to the manufacturer's protocol. GST-ORF13 was used for the pull-down assay. GST-ORF13 or GST alone, immobilized on glutathione-sepharose beads, was incubated with the cell lysates. After pull-down, the proteins were analyzed by western blotting using an anti-c-Myc rabbit antibody (Sigma).

RESULTS AND DISCUSSION

We previously reported that the highest expression of the EseBCD translocon proteins, secreted through the TTSS, occurs when the wild-type strain was incubated at 25 to 30°C in neutral or alkaline pH (Okuda et al. 2009). In this study, we determined whether ORF13 can be secreted into the culture supernatant under the same conditions as for the EseBCD translocon proteins. As shown in Fig. 1B, secretion of ORF13 into the culture supernatant of the wild-type strain was detected from 8 h after incubation. Furthermore,

secretion of ORF13 at 18 h after incubation was detected only in the culture supernatant of the wild-type strain, but not in that of the TTSS-deficient mutant, mET1229, nor the *orf13* mutant (Fig. 1C). These results suggest that ORF13 is secreted through the TTSS under TTSS-inducing conditions. Here, to eliminate the possibility of contamination of the culture supernatant with the cytoplasmic fraction, we carried out the western blot of the cytoplasmic fraction isolated from the wild-type strain, mET1229, and the orf13 mutant, but many non-specific bands with an apparent molecular weight of 8.7 kDa were detected in all samples tested (data not shown). Although we could not completely rule out the possibility of cytoplasmic contamination, we think cytoplasmic contamination is unlikely because ORF13 was not detected in the culture supernatant of mET1229 (Fig. 1C). We will further address this problem in future studies by preparing another peptide antibody against ORF13 that will allow us to probe for ORF13 in the cytoplasmic fraction without non-specific bands. In addition, Lu et al. (2016) reported that ORF13, also known as EscE, is secreted into the culture supernatant in a T3SS-dependent manner, which supports our present data that ORF13 is secreted in the culture supernatant through the TTSS under TTSS-inducing conditions.

To further investigate the role of ORF13 in *Edwardsiella* infection, yeast 2-hybrid screening was used to identify putative binding protein(s). The yeast 2-hybrid screen (1.0×10^6 clones from a mouse brain cDNA library) identified 18 positive clones, in which the cDNA sequences of 14 positive clones were the same as the sequence for secretogranin II (Accession no. NM_009129.2). The remaining 4 positive clones were CUG triplet repeat, RNA binding protein 2 (Cugbp2) (Accession no. NM_010160.1), dynamin I (Accession no. BC034679.1), BAC clone RP23-367M7 from chromosome 3 (Accession no. AC121782.3), and Ena-vasodilator stimulated phosphoprotein (Ena-VASP) (Accession no. XM_979244.1). Among the 5 positive clones, we tested the interaction of ORF13 with secretogranin II, Cugbp2, dynamin I, and Ena-VASP, using a pull-down assay; we excluded BAC clone RP23-367M7 from chromosome 3 since its function is completely unknown. We found that ORF13 interacted only with Cugbp2 under the conditions used (data not shown). Therefore, we focused on Cugbp2, which is associated with apoptosis in cancer cells (Mukhopadhyay et al. 2003, Subramaniam et al. 2008). The interaction of ORF13 with Cugbp2 was confirmed using a pull-down assay: GST-ORF13 bound to Cugbp2 in Hep-2 cell lysates transfected with pCugbp2 (Fig. 2A; Fig. 2B, left panel). On the other hand, GST-ORF13 did not interact with Cugbp2ΔC102 nor Cugbp2ΔC202 (Fig. 2B, center and right panels), which suggests that the C-terminal domain of Cugbp2, corresponding to amino acids 408 to 508, binds to ORF13. Cugbp2 has 2 consecutive RNA recognition motifs (RRMs) in the N-terminal region and another RRM in the C-terminal region (Fig. 2C) (Suzuki et al. 2012). Therefore, ORF13 may interfere with the function of Cugbp2 by binding to the RRM domain in the C-terminal region. The results from the pull-down assay are not sufficient to prove direct binding between ORF13 and Cugbp2, since it is not possible to rule out an interaction mediated by an additional host protein.

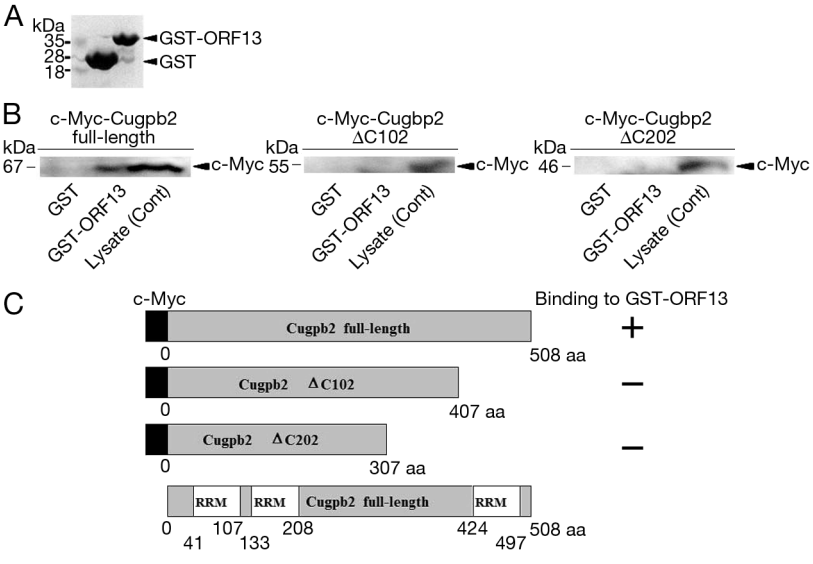

Fig. 2. ORF13 binds to Cugbp2. (A) Coomassie brilliant blue staining of the GST and GST-ORF13 proteins analyzed by SDS-PAGE. (B) Determination of the region of Cugbp2 that mediates binding to ORF13. GST-ORF13 or GST protein alone was immobilized on glutathione-sepharose beads and incubated with the cell lysates containing (left) c-Myc-Cugbp2 full-length, (centre) c-Myc-Cugbp2ΔC102, and (right) c-Myc-Cugbp2ΔC202. After pull-down, proteins were subjected to western blot analysis using an anti-c-Myc antibody. The arrow indicates the presence of c-Myc tagged proteins. Cont: control. (C) Schematic representation of the structure of Cugbp2 and the ORF13-binding domain. Cugbp2 has 2 consecutive RNA recognition motifs (RRMs) in the N-terminal region and another RRM in the C-terminal region (Suzuki et al. 2012). ORF13 binding is a summary of the results shown in (B) and is indicated schematically by minuses and plus

Therefore, future studies will include an alternative GST pull-down assay using recombinant, purified Cugbp2. In this study, we found that ORF13 has the ability to interact with Cugbp2, which is a regulator of the alternative splicing of several transcripts, including α-actinin and the insulin receptor (Suzuki et al. 2012). Cugbp2 is also known as an inducer of apoptosis; siRNA-mediated inhibition of Cugbp2 repressed apoptosis in breast cancer cells in response to radiation injury (Mukhopadhyay et al. 2003, Subramaniam et al. 2008). We previously reported that infection of J774 macrophages with *Edwardsiella tarda* had an anti-apoptotic effect on the macrophages (Okuda et al. 2006), which might be due to binding of ORF13 to Cugbp2. Further studies will be required to determine whether the anti-apoptotic effect of ORF13 on J774 macrophages infected with *E. tarda* occurs through interaction with Cugbp2.

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