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**Salmonella enterotoxin (Stn) regulates membrane composition and integrity**

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**SUMMARY**

The mechanism of action of *Salmonella* enterotoxin (Stn) as a virulence factor in disease is controversial. Studies of Stn have indicated both positive and negative effects on *Salmonella* virulence. In this study, we attempted to evaluate Stn function and its effects on *Salmonella* virulence. To investigate Stn function, we first performed in vitro and in vivo analysis using mammalian cells and a murine ileal loop model. In these systems, we did not observe differences in virulence phenotypes between wild-type *Salmonella* and an *stn* gene-deleted mutant. We next characterized the phenotypes and molecular properties of the mutant strain under various in vitro conditions. The proteomic profiles of the total cell membrane protein fraction differed between wild type and mutant in that there was an absence of a protein in the mutant strain, which was identified as OmpA. By far-western blotting, OmpA was found to interact directly with Stn. To verify this result, the morphology of *Salmonella* was examined by transmission electron microscopy, with OmpA localization being analyzed by immunogold labeling. Compared with wild-type *Salmonella*, the mutant strain had a different pole structure and a thin periplasmic space; OmpA was not seen in the mutant. These results indicate that Stn, via regulation of OmpA membrane localization, functions in the maintenance of membrane composition and integrity.

**INTRODUCTION**

*Salmonella* is a food-borne pathogen that is typically acquired through consumption of contaminated food and water. This bacterium causes severe clinical manifestations, including acute gastroenteritis and typhoid fever (Boyle et al., 2007). Many studies have shown that type III secretion systems, which are encoded within pathogenicity island 1 and 2 (SPI-1 and SPI-2, respectively), are important for the virulence of this organism. These secretion systems are involved in invasion of intestinal epithelial cells and *Salmonella* survival in macrophages (Grassl and Finlay, 2008).

It has been proposed that *Salmonella* enterotoxin (Stn) is a putative virulence factor and causative agent of diarrhea (Chopra et al., 1994; Chopra et al., 1999). Interestingly, it has been shown that the *stn* gene is specifically distributed in *Salmonella* spp. irrespective of their serotypes (Djinjas et al., 1997; Makino et al., 1999; Moore et al., 2007; Lee et al., 2009). This second finding indicates that the *stn* gene might be useful for the identification of detection of *Salmonella* and that Stn might be involved in functions unique to *Salmonella*. Chopra et al. cloned the *stn* gene and showed that it had an enterotoxic activity in a murine ileal loop model (Chopra et al., 1999). Therefore, they proposed that Stn is a *Salmonella* virulence factor and is responsible for the enterotoxicity of *Salmonella*. However, research by other groups did not support this conclusion (Lindgren et al., 1996; Watson et al., 1998; Wallis et al., 1999) and did not show an association of Stn with *Salmonella* virulence. These investigations did not detect differences of virulence phenotypes between wild-type and an *stn* gene-deleted (*Δstn*) mutant. Thus, the role of Stn in *Salmonella* virulence is still debated.

In this study, we examined the relationship between Stn and *Salmonella* virulence using the *Δstn* mutant strain in vitro and in vivo models. Our studies reveal new insights into Stn function in *Salmonella*, suggesting that Stn is involved in the maintenance of membrane composition and integrity.

**RESULTS**

**Role of Stn for the virulence of Salmonella**

To determine whether Stn contributes to *Salmonella* virulence, we examined *Salmonella* virulence in vitro and in vivo using wild-type and *Δstn* mutant *Salmonella*. We did not observe statistically significant differences in invasion ability or intramacrophage survival between wild-type *Salmonella* and the *Δstn* mutant (Fig. 1A,B). Because Chopra et al. showed that Stn exhibits enterotoxic activity in a murine ileal loop model (Chopra et al., 1999), we next investigated the activity of the *Δstn* mutant in that system. The mutant strain still induced fluid accumulation in the ligated murine ileal loop compared with the control loop and behaved in the same manner as a wild-type strain (Fig. 1C).

To investigate the effect of Stn on chemokine transcriptional levels (i.e. RANTES, GM-CSF, MCP-3, CXCL1, CXCL2, CXCL3) in infected HeLa cells, real-time PCR analysis was performed. HeLa...
ompA, we quantified expression of OmpA (Fig. 2A). To determine whether Stn regulates the transcription of another four membrane protein genes, we also did not observe statistically significant differences in the mutant was almost the same as in wild type (Fig. 3). In addition, from these results, we concluded that Stn might regulate membrane integrity by its interaction with OmpA. We next analyzed OmpA localization in the membrane region. From these results, we estimate that Stn modulated chemokine release.

**Characterization of the Δstn mutant to estimate the function of Stn**

To evaluate Stn function, we characterized the phenotypes of the Δstn mutant, including growth under different culture conditions (e.g. pH, temperature, growth in the presence of acid or H₂O₂), motility on a soft agar and antibiotic resistance. No difference was found between these strains (data not shown).

We next analyzed the Salmonella membrane protein fraction by SDS-PAGE. A protein signal was missing in the Δstn fraction and this missing protein was identified as the outer membrane protein A (OmpA) (Fig. 2A). To determine whether Stn regulates the expression of ompA, we quantified ompA mRNA at mid-log phase by real-time PCR. The transcriptional level of ompA in the Δstn mutant was almost the same as in wild type (Fig. 3). In addition, we also did not observe statistically significant differences in the transcription of another four membrane protein genes (ompC, lpp, tamB and ompW) between wild type and the Δstn mutant (Fig. 3). These results indicate that Stn does not affect expression of membrane protein genes, including ompA, and that OmpA localization or levels in the Δstn mutant might differ compared with that of the wild-type strain.

OmpA is a major outer membrane protein of Gram-negative bacteria, including Salmonella, and is associated with many cellular functions (Chai and Foulds, 1997). We next investigated the membrane of Salmonella strains by electron microscopy. Indeed, we found that the Δstn mutant had a different pore structure from a wild-type strain. The Δstn strain also had a very thin periplasmic space compared with a wild-type strain (Fig. 2B, C). From these results, we concluded that Stn might regulate membrane integrity by its interaction with OmpA.

**Stn interacts with OmpA**

As shown in Fig. 2, lack of Stn affects membrane integrity and the localization of OmpA in the outer membrane. From these results, we hypothesized that Stn might interact with OmpA directly to control the localization of OmpA in the outer membrane of Salmonella. To test the hypothesis that OmpA interacts with purified recombinant Stn (named TF-Stn), we carried out far-western blotting. Using purified recombinant OmpA and TF-Stn proteins, we found that membrane OmpA interacted with soluble TF-Stn, which was used as a probe (Fig. 4B). By contrast, a control reaction using TF-tag did not generate a specific signal with OmpA (Fig. 4A). Thus, this result suggests that Stn interacts with OmpA directly, and that it might facilitate the proper localization of OmpA in the organism.

**DISCUSSION**

Although it has been reported that Salmonella produces an agent responsible for enterotoxic activity, little information concerning this factor is available (Finkelstein et al., 1983; Molina and Peterson, 1980). That factor has not been identified thus far. Chopra et al, have shown that Stn from Salmonella typhimurium strain Q1 exhibits enterotoxic and cytotoxic activities (Chopra et al., 1999). Furthermore, they also noted that the deduced amino acid sequence of Stn (amino acid residues 127-142) shows some similarity to the active site of cholera toxin (CT) and heat-labile enterotoxin (LT) ADP-ribosyltransferases (Chopra et al., 1994). Therefore, they proposed that Stn could be a key factor in acute gastroenteritis and diarrhea and could contribute to Salmonella virulence. However, other research groups reported that Stn is not associated with Salmonella virulence, e.g. enterotoxicity, cytotoxic activity...
As mentioned above, the functions of Stn are still debated, but it is interesting to note that the stn gene is distributed only in Salmonella species (Dinjus et al., 1997; Makino et al., 1999; Moore and Feist, 2007; Lee et al., 2009). From this point of view, it is possible that Stn might play a role in unique or special functions of Salmonella. We therefore proposed that biological activities of Stn are important to Salmonella virulence, especially acute gastroenteritis.

In this study, we examined the functions of Stn using the Δstn mutant to verify whether Stn is involved in Salmonella virulence. As shown in Fig. 1, we could not find any differences of virulence phenotypes between the wild type and Δstn. In addition, we could not detect the R-S-EXE triad motif, which is essential for the ADP-ribosyltransferase of CT and LT (Harford et al., 1989; Laing et al., 2011), in the deduced amino acid sequence of Stn (Fig. 5). On the basis of these data, we think that Stn is not an ADP-ribosyltransferase and does not function as a virulence factor of Salmonella.

Previous studies concerning Stn function have been focused on its effect in infected host cells (Chopra et al., 1994; Chopra et al., 1999). By contrast, in this study, we investigated unknown functions of Stn in Salmonella. We attempted to estimate the functions of Stn from its deduced amino acid sequence. It is noteworthy that Stn protein has no homology with other proteins (data not shown). We characterized the Δstn mutant and showed that Stn might affect membrane integrity via regulation of OmpA localization. OmpA is generally synthesized in the cytoplasm in a precursor form with a signal sequence and is translocated into the periplasmic space or outer membrane. Previous studies have indicated that the requirements for translocation of OmpA include the signal sequence and correct protein conformation (Freudl et al., 1985; Freudl et al., 1990). It has been speculated that Stn is located in the cytoplasm, because Stn lacks a typical signal sequence and transmembrane region. Thus, we hypothesize that interaction between Stn and OmpA contributes to the conservation of OmpA protein conformation and might facilitate OmpA translocation into the outer membrane. It is noteworthy that Stn is predicted to be a highly basic protein (isoelectric point (pI) is over 11), whereas OmpA in Salmonella is predicted to be a slightly acidic protein (pI 5.6). Consequently, charge difference might contribute to the interaction between Stn and OmpA. Further detailed studies on the interaction between Stn and OmpA are needed to verify the function of this proposed complex.

Our observations give new insight into Stn function in bacterial cells. Stn might participate in the maintenance or membrane integrity of this bacterium. We are currently underway analyzing the detailed molecular mechanisms by which Stn regulates membrane integrity.

METHODS
Bacteria
Salmonella enterica serovar Enteritidis strain 171, a clinical isolate from Thailand, was used as a standard strain in this study. Bacteria were routinely cultured in Luria-Bertani (LB) medium at 37°C.
Deletion mutant strains were constructed as described previously (Kodama et al., 2002). In brief, a DNA fragment of the stn gene was generated by PCR using primers stn-stn (5′-GGATCCGTGGATCCTGACCTGAAAC-3′) and stn-4 (5′-CTCGAGATTGTCTGTAAGGCTGGA-3′) after the preparation of PCR products using primers stn-1 and stn-2 (5′-ATCGGACCTGCACTCGAGGCTGCAAACGCTGAT-3′) and stn-3 (5′-GGCTTTACCTCAATGCTGATAACGCTGAT-3′) and stn-4, respectively. A DNA fragment of the ompA gene was amplified by PCR using primers omp-1 (5′-CTGCGAGATTGTCCTCCTCAATGCTGATAACGCTGAT-3′) and omp-4 (5′-GCATGATGCCACCAATGACCAG-3′) after the preparation of PCR products using primers omp-1 and omp-2 (5′-AACACTCGGCCACGCCATGACCAG-3′) and omp-3 (5′-AACACTCGGTAGGCTGAGATTGTAAGG-3′) and omp-4, respectively. The PCR products generated using primers stn-1 and stn-4, and omp-1 and omp-4, were cloned into the suicide vector pYAK1, and cloned plasmid was transfected into E. coli strain SM10λpir. After conjugation and disruption by homologous recombination, candidates were confirmed by PCR using primers stn-1 and stn-4 or omp-1 and omp-4, respectively. Resulting mutant strains for the stn and ompA genes were named Δstn and ΔompA, respectively.

Preparation of recombinant proteins

The stn gene was amplified by PCR using primers stn-F (5′-GGATCCCTGGATATGTTGTCGCTG-3′) and stn-R (5′-GTCGCCACCTGCACTCGAGGCTGGA-3′), PCR product was cloned into pCold TF (TaKaRa Bio) and the plasmid construct was transfected into E. coli BL21 (DE3). Recombinant Stn was expressed in LB medium supplemented with 50 μg/ml ampicillin and 1% glucose at 30°C; 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce toxin expression. A fusion protein was generated with the trigger factor (TF)-tag sequence at the N-terminus of Stn (named TF-Stn).

The ompA gene was amplified by PCR using primers omp-F (5′-CGCATGCGCTCCGAAGATAACAC-3′) and omp-R (5′-TAAGCTTTAAGCCTGCAGGCTGATTAC-3′). PCR product was cloned into pQE30 (QIAGEN) and the plasmid construct was transfected into E. coli M15[pREP4]. Recombinant OmpA was expressed in LB medium, supplemented with 100 μg/ml ampicillin, 25 μg/ml kanamycin and 1% glucose at 25°C, by addition of 1 mM IPTG.

Recombinant proteins were purified by Ni Sepharose 6 Fast Flow (GE Healthcare), according to the manufacturer's instructions, under non-denatured (for Stn) and denatured (for OmpA) conditions, with or without 4M urea, respectively.

Preparation of protein fractions

To perform the protein profiles of Δstn cells, we prepared four different protein fractions as follows. To prepare the whole cell protein fraction, bacteria were cultured in LB medium at 37°C for 16 hours with shaking. After washing the bacteria once with PBS and resuspension in 1× loading buffer (10% glycerol, 6.25 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.01 mg/ml bromophenol blue, 5% β-mercaptoethanol), bacterial cells were disrupted by heat for 10 minutes and insoluble proteins were removed by centrifugation at 20,000 g, 4°C for 10 minutes. To prepare the secreted protein fraction, bacteria were cultured in LB medium at 37°C for 20 hours and proteins were precipitated from the supernatant using trichloroacetic acid (final concentration: 5%) at 4°C for 16 hours followed by centrifugation for 1 hour at 20,000 g, 4°C. Pellet was washed twice with ice-cold acetone and neutralized with 1 M Tris-HCl (pH 8.0). The periplasmic protein fraction and membrane protein fraction were prepared as previously described (Sittka et al., 2007).

The concentration of each protein fraction was calculated using a Pierce 660 nm Protein Assay Kit with Ionic Detergent Compatibility Reagent (Thermo Scientific).
incubation continued at 37°C for 10 minutes. After incubation, cells were washed with PBS and incubated at 37°C, 5% CO₂ until 2 hours post infection with 100 µg/ml gentamicin. At 2 hours post infection, medium was replaced with fresh culture medium containing 10 µg/ml gentamicin, and incubation continued until 8 or 24 hours post infection. Infected cells were washed with PBS and lysed with PBS containing 0.1% Triton X-100. Viable bacteria were counted on LB agar plates.

**Electron microscopy and immunostaining of OmpA**

Salmonella morphology was examined by transmission electron microscopy. Bacteria were cultured in LB medium at 37°C for 16 hours and collected by centrifugation (300 g, 10 minutes). Bacteria were fixed in 2% glutaraldehyde buffer (pH 7.4) containing 0.02 M sodium cacodylate, 0.6% NaCl and 0.02% ruthenium red. After fixation in 1.5% osmium tetroxide containing 0.02 M sodium cacodylate and 0.6% NaCl, bacterial cells were dehydrated in ethanol and were saturated with Quetol 653. The sections were prepared by Ultra microtome (Leica Microsystems) and stained with uranyl acetate and citrate.

Immunogold labeling was performed as previously described (Ichinose et al., 2011). In brief, bacteria were cultured on an LB agar plate at 37°C for 16 hours and fixed using 0.2% glutaraldehyde at −80°C. After dehydration, samples were saturated with LR-GOLD resin (Nissin EM) and segments were treated with murine anti-OmpA antibody. Signals were detected using a colloidal gold particle conjugated to anti-mouse IgG (10 nm diameter of conjugated gold; BB International).

All specimens for transmission electron microscopy were examined with a JEM-1230 electron microscope (JEOL).

**Assay for Salmonella enterotoxicity**

Salmonella enterotoxicity assays were performed as described previously, with some modifications (Chopra et al., 1999; Kajikawa et al., 2010). In brief, bacteria were cultured in LB medium at 37°C for 16 hours and washed once with PBS. Prepared bacteria (ca. 1×10⁹ colony forming units (CFU)/loop) were injected into ligated murine ileal loops (C57BL/6 mouse, female, 7-weeks old). At 4 hours after injection, the fluid content of each loop was measured. Activity was expressed as the ratio of the fluid content of the loop (in ml) to its length (in cm).

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted from infected HeLa cells using TRIzol reagent (Invitrogen) and contaminated genomic DNA was removed by DNase I (TaKaRa Bio). cDNA was generated from 1 µg of total RNA using the PrimeScript RT reagent kit and Oligo dT primer (TaKaRa Bio) in accordance with the manufacturer’s instructions. The primer sequences for chemokine genes used in this study were described previously (Okuda et al., 2005; Abdallah et al., 2007; Huang et al., 2007; Okuda et al., 2009; Nishihara et al., 2010). The expression levels of each gene were normalized, with GAPDH as an internal control.

Bacteria were cultured in LB medium at 37°C until mid-log phase (OD₆₀₀=0.8) and total RNA was extracted using TRIzol reagent. cDNA was generated from 1 µg of total RNA using the PrimeScript RT reagent kit and random hexamers (TaKaRa Bio). The primer sequences for membrane protein genes were as follows: ompA (5'-

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**Amino acid sequence analysis**

Proteins (10 µg/lane) were separated by 10% SDS-PAGE and a target protein was excised from the gel. Amino acid sequence was determined by an Agilent 1100 LC/MSD Trap XCT (Agilent Technologies). Protein identification was performed using the Spectrum Mill MS Proteomics Workbench, with a Swiss-Prot protein database search (Ichihara et al., 2006).

**Far-western blotting**

Interaction between Stn and OmpA was analyzed by far-western blotting (Nambu and Kutsukake, 2000). OmpA (1 µg) was loaded on 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Binding of TF-Stn (10 µg) was performed in TBST buffer (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% Tween 20) containing 5% skim milk for 16 hours at 4°C. Signals were generated using anti-TF antibody (TaKaRa Bio) and anti-mouse IgG antibody (Zymed) with ECL Western Blotting Detection Reagents (GE Healthcare), and visualized by LAS-1000 (Fujifilm).

**Invasion assay**

Invasion assays were performed as previously reported (Sittka et al., 2007). HeLa cells (5×10⁵ cells/well in a 12-well plate) were cultured at 37°C under 5% CO₂ for 18 hours in modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences), 2 mM glutamine. Bacteria were added at a multiplicity of infection (MOI) of 10 and centrifuged at 300 g for 10 minutes to synchronize the infection, followed by incubation at 37°C for 1 hour. After infection, cells were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated further for 1 hour at 37°C with 100 µg/ml gentamicin. Invaded bacteria were counted on LB agar plates.

**Survival assay**

Survival assays were performed as described previously (Schwan et al., 2000; Ge et al., 2010). In brief, differentiation of U937 cells (5×10⁵ cells/well) was induced in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 100 µg/ml of gentamicin and 10 mM phorbol 12-myristate 13-acetate (Merck) for 18 hours. Medium was then replaced with fresh RPMI-1640 medium, supplemented with 10% FBS. Before use, medium was replaced again with fresh culture medium and incubation continued at 37°C, 5% CO₂ for 1 hour. Bacteria were added at an MOI of 10 and incubation continued at 37°C for 10 minutes. After incubation, cells were washed with PBS and incubated at 37°C, 5% CO₂ until 2 hours post infection with 100 µg/ml gentamicin. At 2 hours post infection, medium was replaced with fresh culture medium containing 10 µg/ml gentamicin, and incubation continued until 8 or 24 hours post infection. Infected cells were washed with PBS and lysed with PBS containing 0.1% Triton X-100. Viable bacteria were counted on LB agar plates.

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**Fig. 5. Sequence alignment of partial Stn and each of the A subunits of CT and LT.** Amino acid sequences of Stn, CT (CT-A) and LT (LT-A) were derived from S. typhimurium strain Q1, V. cholerae El Tor strain N16961, and enterotoxigenic E. coli strain H10407, respectively (Chopra et al., 1994; Heidelberg et al., 2000; Crossman et al., 2010). Asterisks represent the identical amino acid residues in Stn compared with those of CT and LT. Box and italic letters indicate the conserved motif and catalytic amino acid for ADP-ribosyltransferase of CT and LT (Laing et al., 2011).
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SUPPLEMENTARY MATERIAL
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REFERENCES


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