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RESEARCH ARTICLE

Functional Studies of the Recombinant CdtB, PltA and PltB Subunits from *Salmonella enterica* serovar Javiana

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Abstract

Cytolethal distending toxin (CDT) is a bacterial toxin that causes HeLa cells to arrest in the G2/M phase of the cell cycle, as well as causing cytoplasmic and nucleus distension of most eukaryotic cells. The CdtB subunit, the active subunit of CDT, is a type I deoxyribonuclease. Here we show that the non-typhoidal *Salmonella enterica* serovar Javiana encodes CdtB, PltA and PltB in the same pathogenicity islet. Purified His_6-tagged CdtB, PltA and PltB subunits were tested for their capacity to induce morphological changes in HeLa cells. *S.* Javiana CdtB alone was capable of inducing typical signs of cytolethal distension such as cytoplasmic distension and nuclear enlargement. When added individually, purified PltA and PltB do not exhibit toxic activity, whereas the combination of purified His_6-tagged CdtB with PltA and PltB elicited morphological changes. Purified His_6-tagged CdtB converted supercoiled pUC19 plasmid to relaxed and linear forms, and degrades HeLa cell DNA. These results suggest that the *S.* Javiana CdtB is likely to play an important role during host infection and disease.

INTRODUCTION

Cytolethal distending toxin (CDT) is considered an important factor in intestinal pathogenesis that can cause cell cycle arrest, cytoplasm distension and apoptosis in a broad range of mammalian cells and extend persistence of pathogenic bacteria in the host [1]. CDT inhibits the dephosphorylation of Cdc2 protein kinase by Cdc25, causing cells to continue to grow but not divide, thus producing distended cell morphology [2, 3]. This toxin has been shown to be produced by a variety of Gram-negative bacteria, including *Escherichia coli*, *Campylobacter* spp., *Haemophilus ducreyi*, *Shigella dysenteriae*, *Aggregatibacter actinomycetemcomitans*, and *Salmonella* Typhi [4]. Previously, we have shown that HeLa cells infected with *S.* Javiana wild type were arrested in G_2/M and had distended cytoplasm and nuclei that were larger than those infected with *S.* Javiana Δ*cdtB* and Δ*pltA* strains. The *S.* Javiana Δ*pltB* strain retained the ability to induce cytoplasmic distension and cell cycle arrest, whereas the complemented Δ*cdtB* and Δ*pltAS.* Javiana strains showed activity like the wild type strains [5]. In most bacteria exhibiting CDT activity, the toxin is composed of three subunits, CdtA, CdtB, and CdtC, and their genes are adjacent [6]. In contrast, *S.* Typhi does not encode the CdtA and CdtC subunits but rather has the *cdtB* gene located on a pathogenicity islet, upstream of the *pltA* and *pltB* genes encoding pertussis-like toxins A and B. PltA and PltB

have molecular masses of approximately 25 and 15 kDa respectively, and form acomplex with CdtB (30 kDa) [7]. CdtB, the active subunit, shares structural and functional homology with mammalian deoxyribonuclease I (DNase I) [7]. DNase is an endonuclease that digests single and double stranded DNA by hydrolyzing phosphodiester bonds, suggesting that the CdtB could cause DNA damage when expressed in host cells [1]. In *C. jejuni*, the CdtA and CdtC subunits are responsible for the delivery of the CdtB subunit to thenucleus of the target cell [8]. In *S*. Typhi, CdtB and PltA together act as the active subunit, while PltB contributes to the delivery of the CdtB subunit to the nucleus [7, 9].

Comparative genome sequence analysis of *Salmonella enterica* subspecies *enterica* has revealed two subpopulations, called clade A and clade B [10]. The CDT-islet containing *cdtB, pltA* and *pltB* is present in clade B isolates, including *S*. Javiana, as well as only a small subset of clade A, including *S*. Typhi and *S*. Paratyphi. The CDT-islet found in clade B, *S*. Typhi and *S*. Paratyphi may have been horizontally transferred [10]. According to the Centers for Disease Control and Prevention, in 2001, *S*. Javiana was the fifth most common serotype in the United States and accounted for 3.4% of *Salmonella* isolates [11]. Furthermore, *S*. Javiana is restricted primarily to the Southeast region of the USA [12].Therefore, an understanding of the functionality of the CDT islet in non-typhoidal *Salmonella* is necessary for elucidating disease mechanisms for this serovar.

In this study, we investigate for the first time the functional roles of the purified CdtB, PltA and PltB proteins of a non-typhoidal serovar, *S*. Javiana, with respect to cellular distentionand DNase activity. *S*. Javiana strains isolated from clinical sources were screened for *cdtB, pltA* and *pltB* genes. The roles of these genes for CdtB toxicity were determined by cloning the *cdtB, pltA* and *pltB* genes originating from *S*. Javiana and using purified proteins to examine the biological activity, such as cellular distension and DNase activity, on HeLa cell lines.

2. Materials and Methods

2.1. Bacterial Strains

Fifty isolates of *S*. Javiana were selected for this study. Twenty of these were isolates from foods and environmental samples, and were obtained from the FDA-Arkansas Regional Laboratory (ARL), and thirty isolates, of clinical origin, were obtained from the Arkansas Department of Health (AHD).

2.2. Cell culture conditions

Studies were performed using the HeLa cell line (ATCC-CCL-2). The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated-fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and gentamicin (50 µg/ml, Sigma, St. Louis, MO) at 37 °C in a humidified atmosphere of 5% CO_2. Medium was replenished every 2-3 days and confluent monolayers were treated with 1× trypsin-EDTA (Life Technologies) for further studies. After trpsinization cells were seeded at a density of 1×10^5 cells ml^{-1} into six-well plates (1.5 ml per well). Unless noted otherwise, cells were grown to confluence.

2.3. Construction of plasmids and nucleotide sequence analysis

Oligonucleotide primers were designed (Table 1) to amplify the full length *S*. Javiana *cdtB, pltA* and *pltB* genes, with 5'-*Nco*I and3'-*Xho*I overhangs included for the cloning into His_6-tagged vector pET28a (+) (Novagen, Madison, WI). The nucleotide sequences of the CDT islet genes were determined. Briefly, amplification of the target genes was performed by simplex PCR [13]. The PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Germantown, MD), and after purification the fragments were cloned using the TA CloningKit (Life Technologies) and the recombinant plasmid was transformed into *E.coli* TOP10 according to the manual provided by the supplier. Colonies of transformed *E. coli* were selected, cultured, and used to prepare plasmid DNA. Purified plasmids were sequenced at the University of Arkansas for Medical Sciences, Little Rock, AR. Nucleotide and amino acid sequences were analyzed and compared by using the Lasergene (DNASTAR, Inc., Madison, WI). After analysis of the nucleotide sequence, the TA vectors that carried the inserts *cdtB, pltA, or pltB* were digested with *Nco*I and *Xho*I restriction enzymes (Promega,Madison,WI) and the *cdtB, pltA* and *pltB* inserts were sub-cloned into the pET 28a(+) vector. The recombinant plasmid was transformed into *E. coli* BL21 competent cells and transformants were selected by growing at 37 ºC on LB agar plates supplemented with 30 µg/ml kanamycin (Sigma). The desired recombinants were checked for correct insertion of *cdtB, pltA* and *pltB* by digestion with the restriction endonucleases *Nco*I and *Xho*I.

2.4. Expression and purification of S. *Javiana recombinant CdtB, PltA and PltB proteins*

Recombinant His_6 tagged *cdtB, pltA* and *pltB*were expressed in *E.coli* strain BL21. The recombinant strains weregrown for 5 h at 37 °C in LB broth (500 ml) containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Expression of target genes was assessed by analysis of total cell protein by SDS-PAGE gels with Coomassie blue staining. C-terminal His_6-tagged CdtB, PltA and PltB recombinant proteins were purified from inclusion bodies, using nickel-affinity chromatography under denaturing conditions (6 M GuHCl), and refolded [14]. After centrifugation and filtration, the soluble proteins were tested for biological activity. The proteins were concentrated using Slide-A-Lyzer Concentrating Solution (Thermo Scientific), and the concentrations were determined by using a 2-D Quant kit (GE Healthcare).

2.5. Western blot analysis

Western blot analysis was performed as described previously [15] with slight modifications. Briefly, 1 ml of each IPTG-induced bacterial culture was harvested and centrifuged at 4,000 g for 15 min. The pellets were resuspended in 50 µl of gel loading buffer and heated in a boiling water bath for 5 min. Samples were applied to a 10% polyacrylamide gel. After electrophoresis, proteins in the gel were transferred to a polyvinylidene a difluoride (PVDF) membrane (Immun-Blot® PVDF Membrane, Bio-Rad). The membrane was blocked with 5% skim milk for 1 h at room temperature with shaking. The membrane was incubated with anti-His (C-Term)-HRP antibody (Invitrogen), diluted 1:5000 in 5% skim milk, for 1 h at room temperature with shaking, followed by three washes of 10 min each at room temperature in TBS-Tween buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Protein bands bound by anti-polyhistidine antibody were detected with the Amersham ECL Western blotting detection system (GE Healthcare Life Sciences).

2.6. Cytolethal distending toxin assay

The cytolethal distending activity of the recombinant His_6-tagged CdtB, PltA and PltB proteins was determined as described previously [16, 17] with slight modifications. HeLa cells were seeded into tissue culture plates (6 wells) at a density of $1x10^5$ cells per well in 1.5 ml DMEM. The plates were incubated at 37 ºC in a humidified 5% CO_2 for 22-23 h before treatment with purified proteins. Medium in the 6 well plates was replaced with fresh DMEM + 10% FBS 30 min before the wells were treated with 5 µg/ml of individually purified CdtB, PltA and PltB proteins or mixtures of the proteins, with and without 10 mM $MgCl_2$ and 10mM $CaCl_2$. Plates were incubated for 72 h at 37 ºC in 5% CO_2. After the 72 h treatment the cells were washed 3 times with PBS, fixed with formaldehyde, and observed by light microscopy for demonstration of morphological changes [8].

2.7. DNase assay

DNase activity was assessed as described by Elwell and Dreyfus (2000) with modification. Briefly,1 µg of supercoiled pUC19 plasmid DNA or HeLa cell DNA was incubated with purified His6-tagged CdtB protein (1-3 µg) in a volume of 30 µl of 25 mM HEPES, pH 7.0, 10 mM $MgCl_2$ and 10 mM $CaCl_2$. After 30 min to 2 h incubation at 37 ºC, the reactions were stopped by adding 10 mM EDTA, 6% glycerol and 0.25% bromophenol blue. The samples were loaded directly onto 0.8% agarose gels. Gels were electrophoresed for 30 min at 100 V in 1× TAE (40 mM Tris, 20 mM acetic acid, and 1mM EDTA, pH 8.0) andstained with ethidium bromide. Individual bands were quantified with a Bio-Rad Gel Doc system (Hercules, CA) to view any changes in the electrophoretic mobility of the supercoiled plasmid DNA band.

3.Results

3.1. Cloning expression and purification of CdtB, PltA and PltB

The plasmid constructs containing the His_6-tagged*cdtB, pltA,* and *pltB* are shown in Fig. 1. SDS-PAGE of the cell lysates and purified His_6-tagged proteins are shown in Fig.1A. His_6-tagged CdtB, PltA, and PltB had molecular masses of approximately 29, 25 and 15 kDa, respectively. The presence of induced His_6-tagged protein was confirmed by immunoblot analysis with anti-His_6 antibody (Fig. 1B). No immunopositive bands were detected with the anti-His (C-Term)-HRP antibody on Western blots before induction with1mM IPTG for 5 h at 37 °C (Fig. 1B).

3.2. Cytolethal distending toxin (CDT) assay

HeLa cells were treated with the purified His_6-tagged CdtB, PltA and PltB at 37 ºCand examined for morphological changes to assess the contribution of the individual proteins to cytotoxic effects. After incubation for 72 h with 5 µg of the purified His_6-tagged CdtB/ml, the nuclei and cytoplasm of HeLa cells became overtly distended, compared with the controls (untreated cells) (Fig. 2B). In contrast, cells treated with purified His_6-tagged

PltA or PltB developed a spindle morphology but did not show any detectable distension of either the cytoplasm or the nucleus (Fig. 2C, D). Notoxic effectson were observed when the individual proteins were incubated with HeLa cells in culturesin the absence of $CaCl_2$ and $MgCl_2$ (data not shown).

HeLa cells were also treated with pairs of the purified His_6-tagged proteins. The combination of purified His_6-tagged CdtB with PltA or CdtB with PltB caused distension of the cytoplasm and nucleus, similar to that of the purified His_6-tagged CdtB alone (data not shown). In contrast, adding PltA with PltB in the absence of CdtB did not result in distension of the HeLa cells. Taken together, these results indicated that CdtB was the enzymatically active subunit, and under these conditions did not require the presence of the other subunits for distending activity.

3.3. DNase activity of the recombinant CdtB

The purified His_6-tagged CdtB protein was tested for DNase activity by incubating several concentrations with supercoiled pUC19 plasmid DNA or HeLa cell DNA for various times (30 min, 1 h and 2 h) at 37 °C in the presence or absence of $CaCl_2$ and $MgCl_2$. The CdtB exhibited DNase activity against both the plasmid DNA and HeLa cell DNA in the presence of $CaCl_2$ and $MgCl_2$ (Fig. 3), but showed no DNase activity in the absence of $CaCl_2$ and $MgCl_2$ (data not shown). This DNase activity resulted in the conversion of supercoiled plasmid DNA to the relaxed and linear product, in the reaction containing 3 µg of purified His_6-tagged CdtB, as soon as 30 min after initiation of incubation (Fig. 3A). After a 2 h incubation of purified His_6-tagged CdtB with supercoiled plasmid DNA or HeLa cell DNA the substrate was completely degraded (Fig. 3C, D).These results suggest that CdtB of *S.* Javiana has a DNase activity similar to that of members of the mammalian DNase I family.

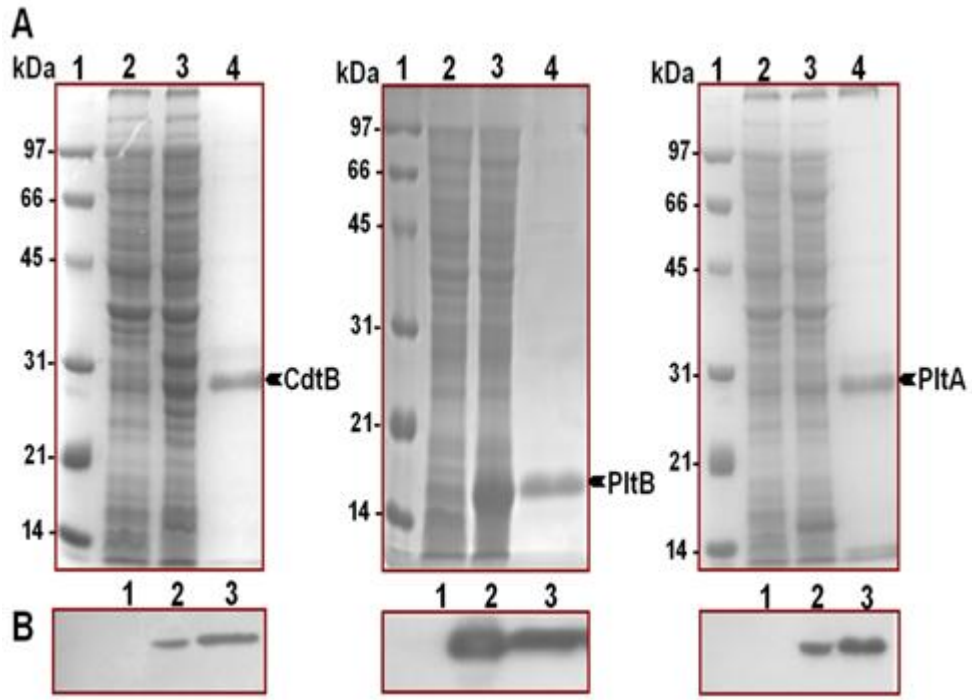

Figure 1. SDS-PAGE and immunoblot analyses of purified recombinant His_6-tagged CdtB, PltA and PltB proteins. A. Coomassie brilliant blue staining of molecular weight markers (lane 1), whole cell lysates without IPTG induction (lane 2), whole cell lysates from IPTG-induced samples (lane 3), and purified Cdt or Plt proteins (lane 4). B. Immunoblot analysis of the regions of SDS-PAGE gels containing the induced His_6-tagged proteins. Lane 1 contains the uninduced whole cell lysate, lane 2 contains the IPTG-induced whole cell lysate, and lane 3 contains the purifiedHis_6-tagged protein.

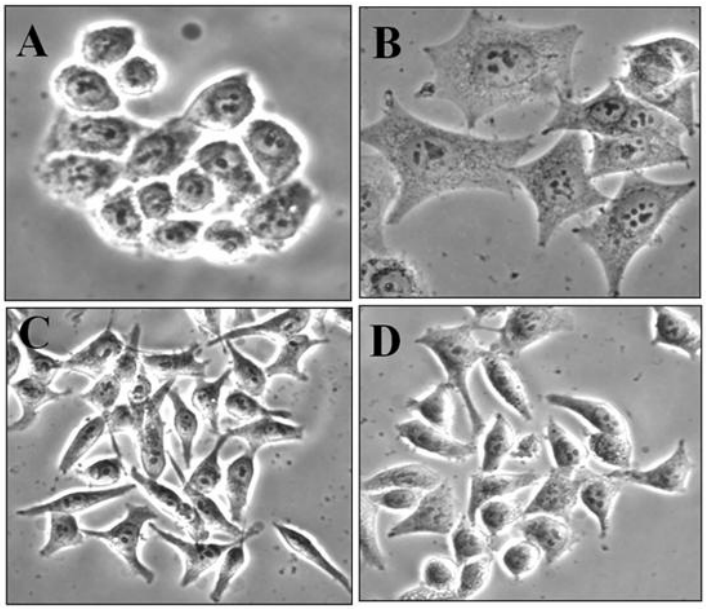

Figure 2. Effects of purified CdtB, PltA and PltB proteins on HeLa cells. Purified His_6-tagged protein was added at a concentration of 5 µg/ml to cultured HeLa cells. 72 h after treatment, the cells were examined by light microscopy. All images are at the same magnification (40×) of the light microscope. A: non infected cells; B: Cells treated with purified His_6-tagged CdtB; C: Cells treated with purified His_6-tagged PltA; D: Cells treated with purified His_6-tagged PltB.

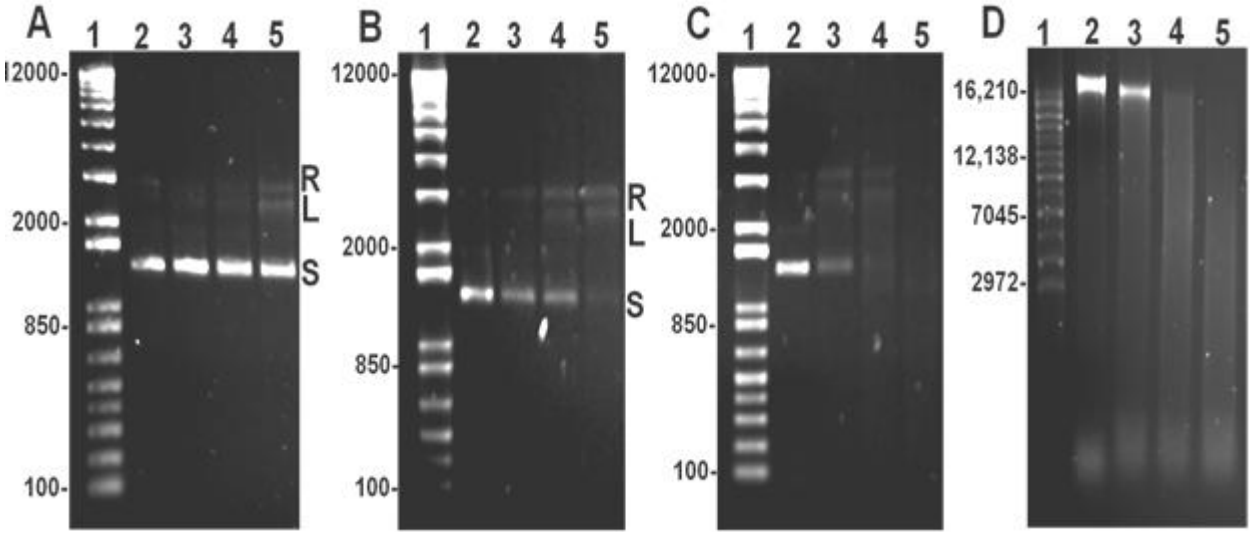

Figure 3. DNase activity of purified His_6-tagged CdtB. In panels A, B, and C, CdtB was incubated with supercoiled plasmid (pUC19) DNA at 37ºC in the presence of Ca^{2+} and Mg^{2+} for 30 min (A), 1 h (B), and 2 h (C). Lane 1 is the molecular weight marker; Lane 2 is the plasmid with no CdtB added;and Lanes 3-5 have 1 µg, 2 µg, and 3 µg of CdtB, respectively. In panel D, HeLa cell DNA was incubated with 3 µg CdtB for various times. Lane 1 is the molecular weight marker, lane 2 is the HeLa cell DNA with no CdtB, and lanes 3-5 show CdtB incubation for 30 min, 1 h, and 2 h, respectively.

TABLE

Table 1. Primers sequences for PCR amplification of *cdtB*, *pltA* and*pltB* genes

Gene	Sequence of Nucleotides	Size (bp)	Function of gene	Reference
cdtB	F- GCCATGGAAAAACCTGTTTTTTTCCTTCTG R- GCTCGAGACAGCTTCGTGCCAAAAAGGCTA	810	Host recognition Invasion	This study
pltA	F- GCCATGGAAAAGTTAATATTCTTAACCTTAT R- GCTCGAGTTTAGAAAGTATAAGTTCTATTAC	729	Pertussis like toxin A, Delivery of CdtB	This study
pltB	F- GCCATGGATATAAATAAGTTTGTGCCTGTTT R- GCTCGAGCTTGGGTCCAAAGCATTGTGTCGC	414	Pertussis like toxin B, Delivery of CdtB	This study

4. Discussion

Cytolethal distending toxin (CDT) has been found in several Gram-negative bacterialpathogens [18].This toxin can inducecytoplasmic distension andextend bacterial persistence in several kinds of mammalian cells [10, 19, 20].CDT has been detected among *Salmonella* serovars, including *S*. Typhi. In this intracellular pathogen, *cdtB* is not co-located with the *cdtA* and *cdtC* genes, and CdtB does not require CdtA and CdtC for cytolethal distending activity. However, the toxicity of *S*. Typhi CDT requires two genes, *pltA* and *pltB*, to induce signs of intoxication, such as cellular distension and cell cycle arrest in eukaryotic cells [7].

To investigate each of the CDT subunits and the possible interactions between them in inducing signs of intoxication in cell lines, we cloned the *cdtB*, *pltA* and *pltB* genes from *S*. Javiana and purified the recombinant His_6-tagged CdtB, PltA and PltB proteins. We showed that the recombinant reconstituted *S*. Javiana CdtB, PltA and PltB subunits were similar in size to those of CDT (CdtB, PltA and PltB) of *S*. Typhi [7]. In previous studies in *S*. Typhi, two or all three CDT subunits were required for toxic activity in target cells [2, 7, 8, 21]. However, we found that the purified His_6-tagged CdtB from *S*. Javiana induced cytoplasmic distension andnuclear enlargement in HeLa cells in the absence of the PltA and PltB proteins. This result was comparable to those previously reported for rCdtB purified from *E. coli* transformed with a C-terminal His_6-tag plasmid containing the *cdtB* gene of *Aggregatobacter actinomycetemcomitans* [22, 23]. These observations were consistent with the experiments of Lara-Tejero and Galan in which microinjection of purified *C. jejuni* CdtB into the cytoplasm of cultured COS-7 cells caused morphological changes [24]. Therefore, our data support the hypothesis that CdtB is the biologically active subunit of CDT. However, Spano et al., found that *S*. Typhi CdtB required the presence of both PltA and PltB for cytotoxicity in intestinal epithelial cells [7].

Several studies have reported that CDTs bind to the host cell surface and enter the cell by receptor mediated endocytosis [25, 26], Although Corts-Bratti et al. have suggested that CDT is internalized by endocytosis within clathrin coated vesicles [27]. The toxins then gain access to an interacellular compartment. In most cases, proteins smaller than 40 kDa can diffuse freely through the nuclear pore complex to eventually reach the nucleus [28]. However, some protein toxins may reach intracellular compartments by engaging by the retrotranslocation machinery [6]. Clearly, further investigation is required toidentify CDT receptors and mechanisms of toxin transport into host cell.

CdtB is a mammalian-like DNase I that causes DNA damage within the target host nucleus [24, 29]. When the recombinant His_6-tagged CdtB from *S*. Javiana was tested for DNase activity, we found that this CdtB indeed possessed DNase activity when incubated with DNA in the presence of Ca^{2+} and Mg^{2+}, but not when incubated in the absence of either Ca^{2+} or Mg^{2+}. This is consistent with previous studies that CdtB may require Ca^{2+} and Mg^{2+} for nuclease activity [30-32].

Our observations that *S. enterica* serovar Javiana CdtB exhibits DNase activity and can act alone to induce cytoplasmic and nuclear enlargement is an important step in elucidating the intracellular biology of *S*. Javiana, as well as providing insights into the pathogenesis of disease causedby this important serovar of *S. enterica*. The characterization of CDT from non-typhoidal *Salmonella* in an *in vivo* pathogenesis model will bring additional insights into the biology of *Salmonella* infection in a variety of animal hosts.

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