Delivery of a *Salmonella* Typhi Exotoxin from a Host Intracellular Compartment

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SUMMARY

*Salmonella* Typhi, an exclusive human pathogen and the cause of typhoid fever, expresses a functional cytolethal distending toxin for which only the active subunit, CdtB, has been identified. Here, we show that PltA and PltB, which are encoded in the same pathogenicity islet as *cdtB*, associate with CdtB to form a multipartite toxin. PltA and PltB are homologs of components of the pertussis toxin, including its ADP-ribosyl transferase subunit. We also show that PltA and PltB are required for the delivery of CdtB from an intracellular compartment to target cells via autocrine and paracrine pathways. We hypothesize that this toxin, which we have named “typhoid toxin,” and its delivery mechanism may contribute to *S. Typhi*’s unique virulence properties.

INTRODUCTION

Typhoid fever caused by *Salmonella enterica* serovar Typhi (S. Typhi) results in ~200,000 annual deaths world-wide, mostly in developing countries where infections are endemic (Ivanoff et al., 1994). Unlike other *Salmonella enterica* serovars, S. Typhi can only infect humans, causing life-threatening systemic infections as well as persistent, often life-time colonization by establishing residence most commonly within the gall bladder (House et al., 2001; Parry et al., 2002). Despite its public health importance, relatively little is known about the virulence factors that confer on this bacterium its unique pathogenicity. Comparison of the nucleotide sequence of the genome of *S. Typhi* with those of other *S. enterica* serovars has shown the presence of an unusually large number of pseudogenes in this human adapted serovar (Deng et al., 2003; Edwards et al., 2002; Parkhill et al., 2001). This observation has led to the proposal that the reduction of its functional genome may be at least in part responsible for *S. Typhi*’s narrow host range. Genome comparison has also revealed the presence of a small number of putative virulence genes that are unique to this serovar or that have very limited distribution among other *S. enterica* serovars and, therefore, may contribute to its distinctive virulence properties. One of these genes encodes CdtB, a homolog of the active or “A” subunit of the cytolethal distending toxin (CDT) (Haghjoo and Galán, 2004). This toxin, which is present in several bacterial pathogens, causes cell-cycle arrest and cellular distension because it inflicts DNA damage on the intoxicated cells (Lara-Tejero and Galán, 2000, 2002). Unlike other bacteria that encode CDT, *S. Typhi* does not encode homologs of the “B” subunits of CDT (i.e., CdtA and CdtC), which mediate toxin delivery into target cells. However, *S. typhi* CdtB can induce DNA damage when it is transiently expressed or microinjected into the cytosol of mammalian cells (Haghjoo and Galán, 2004). More importantly, *S. Typhi* possesses cytolethal distending toxicity, and CdtB is delivered by an unknown pathway that is strictly dependent on bacterial internalization into host cells (Haghjoo and Galán, 2004). In fact, *cdtB* is unusual in that it is only expressed by intracellularly localized *S. Typhi*.

Here, we report that two proteins, PltA and PltB, which are encoded within the same pathogenicity islet that carries *cdtB*, exhibit amino acid sequence similarity to the pertussis toxin “A” subunit and one of the components of its heteropentameric “B” subunit, respectively. Like *cdtB*, expression of *pltA* and *pltB* is induced intracellularly. Remarkably, we found that PltA and PltB form a complex with CdtB and are required for cytolethal distending toxicity by aiding the delivery of CdtB through autocrine and paracrine pathways.

RESULTS

*S. Typhi* Encodes Homologs of the Pertussis Toxin that Are Expressed by Intracellular Bacteria

*cdtB* is encoded within a pathogenicity islet that is unique to *S. Typhi*. Closer inspection of this islet revealed the presence of two open reading frames capable of encoding polypeptides with significant amino acid sequence similarity to the pertussis toxin ADP-ribosylating “A” subunit and just one of the 5 components of its heteropentameric “B” subunit (Kaslow and Burns, 1992) (we named these genes *pltA* and *pltB* for pertussis-like toxin A and B, respectively) (Figure 1A and Figure S1 available with this article online). Purified PltA was able to ADP-ribosylate a protein in host cell lysates, although PltA[133A], a derivative carrying a mutation in a residue predicted to be important for catalysis, did not (Figure S2). These results suggest that PltA is an active toxin. The absence of homologs of critical components of the pertussis toxin B subunit was reminiscent of *S. Typhi* CDT, which, as discussed above, also lacks the components of its B subunit. Since *cdtB* is expressed only after bacterial internalization into host cells (Haghjoo and Galán, 2004), we tested whether *pltA* and *pltB* expression was also induced once *S. Typhi* reached an intracellular compartment. We found low expression of the *pltBA* operon when *S. Typhi* was grown in standard...
bacterial culture medium (Figure 1B). However, like cdtB, transcription of pltBA was induced after infection of cultured intestinal epithelial cells (Figure 1B). Consistent with this observation, epitope-tagged PltB was not detected after up to 3 hr of infection of cultured intestinal epithelial cells (Figure 1C). Eight hours after infection, staining in the form of puncta radiating from the S. Typhi containing vacuole was also noticeable. By 24 hr of infection, the PltB-stained puncta, which we hypothesize represent toxin that has left the bacteria-containing vacuole, were readily seen throughout the cell (Figure 1C). Taken together, these results showed that, like cdtB, the expression of pltA and pltB is induced within host cells and suggest that the toxin can exit the bacteria-containing vacuole to localize in puncta-like structures.

**PltA and PltB Are Required for CdtB Delivery from an Intracellular Compartment**

We tested the hypothesis that PltA and PltB are required for CdtB secretion from the bacterial cell. Indeed, other multisubunit toxins, such as pertussis toxin, are known to require specific secretion systems to export the folded and assembled toxin from the bacterial periplasm to the extracellular space (Burns, 2003; Farizo et al., 2000). However, this does not seem to be the case since equivalent levels of CdtB were found in detergent-soluble lysates (which represent the secreted fraction of the toxin) of cells infected with either wild-type or the DpltA or DpltB mutants, although CdtB(D1-22), a mutant lacking the predicted secretion signal, was not found in the lysates (Figure 3A). We
showed CdtB staining in association with the bacterial surface and throughout the cell after 24 hr of infection (Figure 3B). Differ-

Contrast, cells infected with the ΔpltA or ΔpltB mutant strains showed CdtB staining in association with the bacterial surface

but never associated with puncta, even after 24 hr of infection (Figure 3D). The distribution of CdtB in puncta throughout the cell was restored upon introduction of a plasmid encoding pltBA (Figure 3D). These results indicate that the lack of cytolethal distending toxicity in the ΔpltA or ΔpltB mutants is not due to a defect in CdtB secretion from the bacteria but presumably from its failure to exit the Salmonella-containing vacuole and reach its place of action.

CdtB, PltA, and PltB Form a Complex
In Vitro and Colocalize within Host Cells
The functional relationship between PltA, PltB, and CdtB suggested the possibility that these proteins form a complex that may be required for the delivery of CdtB to its place of action. We, therefore, tested whether PltA, PltB, and CdtB could form a complex in vitro. Polyhistidine-tagged versions of these proteins were expressed in E. coli, purified from inclusion bodies, refolded in vitro, and tested for their ability to form a complex by gel filtration or ion exchange chromatography. When refolded together and applied to a gel filtration column, PltA and PltB eluted with a profile that indicated the formation of a complex (Figure 4A). The ability of CdtB to interact with the PltA/PltB complex was tested by ion exchange chromatography. When applied by itself to a MonoQ ion exchange column, CdtB was not retained and was recovered in the flow-through fraction (Figure 4B). Under the same running conditions, the PltA/PltB complex was retained in the MonoQ column (data not shown). Addition of CdtB to the PltA/PltB complex resulted in the retention of CdtB in the column, indicating that the three proteins form a complex (Figure 4B). We then tested whether CdtB colocalizes with PltA/PltB during bacterial infection. Henle-407 cells were infected with a S. Typhi strain encoding 3× -FLAG epitope-tagged PltB and a 3× -Myc epitope-tagged CdtB, and the localization of these proteins was investigated by fluorescence microscopy. As shown in Figure 4C, CdtB and PltB colocalized both on the bacterial surface and in puncta throughout the cell. Taken together, these results indicate that, consistent with their functional relationship, CdtB forms a complex with PltA and PltB and traffics through the cell presumably in association with these proteins.

CdtB Is Exported to the Extracellular Medium
in a PltA/PltB-Dependent Fashion
We found that infection of cultured epithelial cells with S. Typhi resulted in cytolethal distending intoxication of virtually all cells despite the fact that only a fraction of the cells were actually infected. This observation suggested the possibility that CdtB is transported to the culture medium, intoxicating cells in a paracrine fashion. We, therefore, examined whether CdtB toxic activity could be detected in the culture medium of infected cells. Cells were infected with wild-type S. Typhi, and 24 hr after infection, the presence of cytolethal distending activity in the bacteria-free culture medium was examined by applying cell culture supernatant filtrates to uninfected cells. Cells that were treated with a culture filtrate of cells that had been infected with wild-type S. Typhi showed clear signs of intoxication such as cellular distension and cell-cycle arrest (Figure 5). In contrast, cells treated with a culture filtrate obtained from cells infected with the S. Typhi ΔpltA, ΔpltB, or ΔcdtB mutant strains showed no signs of toxicity (Figure 5). Consistent with the presence of toxin

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Figure 2. PltA and PltB Are Required for CdtB Toxicity
Effect of ΔpltA and ΔpltB on cytolethal distending toxicity. Henle-407 cells were infected with different S. Typhi strains as indicated, and 72 hr after infection, the cells were examined by phase contrast microscopy (insets) or processed to measure DNA content by flow cytometry as described in the Experimental Procedures. The peaks corresponding to cells in G0–G1, S, or G2 are indicated. Equivalent results were obtained in several repetitions of the same experiment. Scale bars, 50 µm.
activity in the infection medium, both CdtB and PltB were detected by western blot analysis of filtrates obtained from infected cells (Figure S6). The presence of the toxin in the cultured supernatant was not likely due to lysis of the infected cells since the total number of cells and, more specifically, the number of infected cells did not change over the course of the experiment (Figure S7). Furthermore, the amount of toxic activity recovered from cell culture supernatants could not have originated from the very small number of cells that could have lysed and escaped detection with our assays. In addition, toxicity of the ΔpltA mutant was not rescued by the addition of purified PltA to the infection medium (Figure S8) and, as shown above, CdtB did not appear in puncta in cells infected with the ΔpltA or ΔpltB mutant strains (see Figure 3D). Taken together, these results indicate that CdtB is transported from its site of production within the host cell to the extracellular medium and that PltA and PltB are essential for this transport.

**CdtB Intoxicates Cells via Autocrine and Paracrine Delivery Pathways**

The presence of CdtB-dependent cytolethal distending toxicity in the culture medium suggested the presence of a potential paracrine mechanism by which the exported toxin may intoxicate uninfected neighboring cells or may even reach systemic sites to intoxicate cells from other tissues. To investigate this possibility, we infected cells with wild-type S. Typhi at a multiplicity of infections such that only ~60% of the cells were infected. Subsequent to infection, we added a toxin-neutralizing antibody, and at 48 hr postinfection, we examined cells for signs of cytolethal distending intoxication. Addition of the neutralizing antibody completely blocked the distension and cell-cycle arrest of uninfected cells. Surprisingly, however, addition of the neutralizing antibody also blocked the distension and cell-cycle arrest of infected cells (Figure 6A) as well as the ability of CdtB to induce DNA damage (as measured by the detection of phosphorylated DNA in the infection medium, both CdtB and PltB were detected by western blot analysis of filtrates obtained from infected cells (Figure S6). The presence of the toxin in the cultured supernatant was not likely due to lysis of the infected cells since the total number of cells and, more specifically, the number of infected cells did not change over the course of the experiment (Figure S7). Furthermore, the amount of toxic activity recovered from cell culture supernatants could not have originated from the very small number of cells that could have lysed and escaped detection with our assays. In addition, toxicity of the ΔpltA mutant was not rescued by the addition of purified PltA to the infection medium (Figure S8) and, as shown above, CdtB did not appear in puncta in cells infected with the ΔpltA or ΔpltB mutant strains (see Figure 3D). Taken together, these results indicate that CdtB is transported from its site of production within the host cell to the extracellular medium and that PltA and PltB are essential for this transport.

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Histone H2AX in the nucleus of infected cells; Lowndes and Toh, 2005) (Figure 6B). However, the presence of the CdtB-associated puncta in infected cells was not affected by the addition of the neutralizing antibody (Figure 6C), indicating that addition of the antibody did not block the production and intracellular transport of the CdtB toxin. These results indicate that the autocrine and paracrine pathways are presumably the only mechanisms of delivery of CdtB into cells, and argue against a mechanism of delivery from the Salmonella-containing vacuole to the site of action in the nucleus without an extracellular stage.

**DISCUSSION**

Symbiotic and pathogenic bacteria that have sustained a prolonged association with their hosts have evolved very complex functional interfaces. Often, central to the shaping of these functional interfaces are bacterial proteins that have the capacity to...
modulate cellular functions, such as cytoskeleton dynamics, vesicular trafficking, cell-cycle progression, and innate immune responses. Bacteria have evolved a variety of strategies to deliver those proteins to their site of action. In the simplest form, the bacterial proteins or toxins have, in their primary amino acid sequence, all the information necessary not only to modulate cellular functions but also to reach their destination within the target cell (Just and Gerhard, 2004). In other instances, the help of

Figure 6. CdtB-Mediated Intoxication Occurs through PltA/PltB-Dependent Autocrine and Paracrine Delivery Pathways

(A) Exogenous application of a CdtB-neutralizing antibody inhibits CdtB-mediated intoxication after bacterial infection. Henle-407 cells were infected with wild-type S. Typhi or a cdtB mutant (as a control), and 4 hr after infection, CdtB neutralizing antibody or the preimmune serum were added. Fifty-two hours after infection, the cells were examined by phase-contrast microscopy (insets) and processed to measure DNA content by flow cytometry as indicated in the Experimental Procedures. The peaks corresponding to cells in G0-G1, S, or G2 are indicated. Equivalent results were obtained in several repetitions of this experiment. Scale bar, 20 μm.

(B) Exogenous application of a CdtB-neutralizing antibody inhibits CdtB-mediated DNA damage. Henle-407 cells were infected with wild-type S. Typhi or a cdtB mutant in the presence or absence of a CdtB neutralizing antibody, and 30 hr after infection, cells were stained with a mouse monoclonal antibody directed to phosphorylated histone H2AX (green) to identify foci of DNA damage, a rabbit polyclonal antibody to S. Typhi (red), and DAPI (blue) to stain DNA. Scale bar, 20 μm.

(C) Exogenous application of a CdtB-neutralizing antibody does not alter the intracellular distribution of CdtB. Henle-407 cells were infected with a S. Typhi strain encoding 3x-FLAG epitope-tagged CdtB, and 4 hr after infection, CdtB neutralizing antibody or the preimmune serum (as indicated) were added. Twenty-four hours after infection, cells were stained with a mouse monoclonal antibody directed to the FLAG epitope (green), a rabbit antibody directed to S. Typhi (red), and DAPI (blue) to stain DNA. Scale bar, 5 μm.

Figure 7. Exogenous Addition of the CdtB/PltA/PltB Complex Results in Cytolethal Distending Toxicity

(A) Henle-407 cells were treated with 1 μg/ml total protein of purified CdtB, PltA, or PltB in different combinations as indicated, and 72 hr after treatment, the cells were examined under a phase microscope (insets) or processed to measure DNA content by flow cytometry as indicated in the Experimental Procedures. The peaks corresponding to cells in G0-G1, S, or G2 are indicated. Equivalent results were obtained in several repetitions of the same experiment. Coomassie-blue-stained SDS-PAGE of the preparations used in these studies are shown in each panel.

(B) Model for CdtB/PltA/PltB toxin delivery. After internalization (I), S. Typhi reaches a compartment where expression of cdtB, pltA, and pltB can take place (II). The CdtB/PltA/PltB complex is secreted from the bacteria into the lumen of the S. Typhi-containing vacuole, and it is recognized and packaged into transport carriers (III). The complex is then transported to the plasma membrane and secreted to the extracellular medium (IV) from where it can target susceptible noninfected cells (e.g., cells of the immune system) and induce DNA damage (V). Infected cells that do not express a receptor for the toxin would be resistant to the toxin and provide a safe haven for the bacteria (IV and V).
another protein or proteins (i.e., the “B” subunit) is necessary to deliver the enzymatically active bacterial protein (i.e., the “A” subunit) into the appropriate target cell (Aloulf, 2000). Other delivery mechanisms utilize complex multiprotein machines (e.g., type III and type IV protein secretion systems) to deliver groups of bacterial proteins to the appropriate target cell in a temporally and spatially coordinated manner (Cascales and Christie, 2003; Galán and Wolf-Watz, 2006). Here, we have described a mechanism that may help an intracellular pathogen to deliver a toxin to systemic sites.

We have previously reported that S. Typhi encodes the “A” subunit homolog of the cytolethal distending toxin (CDT) but does not encode homologs of the “B” subunit components (Haghjoo and Galán, 2004). We also showed that cytolethal distending intoxication requires bacterial internalization into host cells. We have described here that in the same pathogenicity islet, S. Typhi also encodes an active ADP-ribosyl transferase that is a homolog of the pertussis toxin ADP-ribosylating “A” subunit (PltA). Interestingly, S. Typhi encodes just one homolog (PltB) of the five components of pertussis toxin heteropentameric “B” subunit. The presence of an “A” subunit homolog of a toxin with the absence of “B” subunit counterparts is reminiscent of S. Typhi CDT, which as discussed above, lacks homologs of its “B” subunit. Remarkably, we found that PltA/PltB is required for CdtB-mediated toxicity. Our studies indicate that such a role is independent of the enzymatic activity of PltA since a catalytic mutant of this toxin was competent for the delivery of CdtB. Rather, our results suggest that CdtB must associate with PltA/PltB to be engaged by the cellular transport machinery that eventually delivers this complex to the extracellular medium, presumably in a common transport carrier. Remarkably, we found that transport to the extracellular space is essential for CdtB intoxication, which occurs exclusively through autocrine or paracrine pathways. These conclusions are supported by the following observations: (A) CdtB colocalizes with PltA/PltB in structures visualized as puncta by immunofluorescence, (B) CdtB-associated puncta most likely represent vesicles since they have a measurable buoyant density and can protect CdtB from protease treatment unless in the presence of detergent, (C) CdtB, its associated cytolethal distending toxicity, and PltA/B are found in the extracellular medium of infected epithelial cells, (D) addition of a neutralizing antibody effectively blocks toxicity both in infected and uninfected cells, and (E) in the absence of pltA or pltB, there is no CdtB-associated toxicity, no CdtB-associated puncta within cells, and no toxicity in the extracellular medium.

We also found that both PltA and PltB are essential for CdtB delivery from the extracellular space since the CdtB/PltA/PltB complex, but not CdtB alone or in combination with either PltA or PltB, was readily toxic when added exogenously. Addition of the CdtB/PltA/PltB complex, however, did not lead to the detection of toxin-containing puncta in intoxicated cells (data not shown), suggesting that the CdtB/PltA/PltB-associated puncta represent “outgoing” (i.e., on its way to the extracellular medium) but not “incoming” (i.e., coming from the extracellular space) toxin. Consistent with this hypothesis, addition of the neutralizing antibody did not block the formation of toxin-associated puncta, although it completely blocked intoxication.

In essence, CdtB/PltA/PltB constitutes a tripartite exotoxin that we have named “typhoid toxin.” This toxin has two distinct biochemical activities, a DNase activity associated with CdtB and an ADP-ribosylating activity associated with PltA. We hypothesize that evolution may have reshaped what may have originally been two independent toxins to combine them into one single tripartite toxin that is suited for its delivery from an intracellular location, and hence adapted to the biology of an intracellular pathogen. According to the model suggested by our results (Figure 7B), after synthesis by intracellular bacteria, this toxin is subsequently exported to the extracellular space from where, in an autocrine and paracrine fashion, it can intoxicate not only bacterially-infected cells but also neighboring cells or even cells at systemic sites if transported to such locations. What advantages might this unusual delivery pathway confer to S. Typhi? Unlike other S. enterica serovars, S. Typhi is known to persistently infect humans for extended periods of time and, if untreated, potentially for life. It is thought that S. Typhi establishes residence within an intracellular compartment (presumably in the gall bladder) although the specific cell type(s) in which it may reside has not been defined (House et al., 2001; Parry et al., 2002). However, according to the model presented here, it is possible that infected cells lacking receptors for the toxin could provide a safe haven for the bacteria and at the same time serve as a source of toxin that could act on other cells and at systemic sites. Although the targets of the ADP-ribosylating activity of PltA are unknown, CDT through the action of its active subunit CdtB, is known to hamper the activity of immune cells (Lara-Tejero and Galán, 2002; Mooney et al., 2001; Shenker et al., 1999). Therefore, the typhoid toxin activities, coupled to its unique delivery mechanism, may be critical for S. Typhi to establish persistent infection in the human host and avoid immune defenses.

More studies will be required to clarify the mechanisms of toxin transport out of and into the cell, as well as to identify the cellular machinery involved in these steps. However, our results highlight the potentially dynamic nature of the S. Typhi-containing vacuole, which does not seem to remain isolated from other cellular compartments but instead seems to be permissive for the delivery of bacterial products to other cellular compartments. In fact, such a dynamic behavior of a bacteria-containing vacuole has been previously reported for Mycobacterium tuberculosis, although in this case it was shown that bacterial lipids are transported outside of the bacteria-containing phagosome (Beatty et al., 2000). The dynamic nature of bacteria-containing phagosomes may have important implications for the interaction of intracellular microbial pathogens with the innate and acquired immune systems.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Bacterial Strains, Media, and Growth Conditions**

The wild-type Salmonella enterica serovar Typhi strain ISP2825 has been described previously (Galán and Curtiss, 1991). Mutant strains carrying in-frame deletions or insertion mutations in cdtB, pltA, or pltB strains expressing chromosomally encoded 3×FLAG or 3×Myc tagged versions of these proteins or carrying the transcriptional reporter fusion pltA::lux in the chromosome were constructed by standard recombinant DNA and allelic exchange procedures as previously described (Kaniga et al., 1994). The pltA and pltB complementing plasmid was constructed by cloning a 1.6 kb DNA fragment comprising both open reading frames and 500 bp upstream of pltB, which includes the promoter region, in the BamHI site of the pACYC184 vector (Chang and...
Cell Host & Microbe
Exotoxin Delivery by an Intracellular Pathogen

Cohen, 1978), Plasmids expressing polyhistidine-tagged ptA, ptB, and cdTB were constructed by cloning the PCR-amplified genes into the pQE-60 vector (Qiagen), ptA\textsuperscript{E126A}, which carries a single amino acid substitution within its catalytic site, was generated using the quick-change system (Stratagene) following the manufacturer’s instructions. S. Typhii strains were grown in LB broth containing 0.3 M NaCl at 37°C. When required, kanamycin (50 μg/ml), ampicillin (100μg/ml), and chloramphenicol (20 μg/ml) were added.

Cell Culture, Bacterial Infections, and Toxicity Assays
Henle-407 intestinal epithelial cells were grown in DMEM supplemented with 10% bovine calf serum (BCS; HyClone). Overnight cultures of the different S. Typhii strains were diluted 1/20 in LB broth containing 0.3 M NaCl and grown until they reached an optical density measured at 600 nm (OD\textsubscript{600}) of 1.0. Cells were infected with the different strains of S. Typhii at a multiplicity of infections of 30 in HBSS. One and a half hours postinfection, cells were washed three times with HBSS and incubated for 1 hr in DMEM supplemented with 10% BCS and gentamicin (100 μg/ml) to kill extracellular bacteria. Cells were then washed and fresh DMEM containing BCS and 5 μg/ml gentamicine was added to avoid cycles of reinfection. When appropriate, neutralizing or preimmune serum was added 4 hr after infection at a dilution of 1:50. Cells were monitored for up to 3 days by phase-contrast microscopy for signs of toxicity orwere processed for cell-cycle analysis as previously described (Lara-Tejero and Galán, 2000, 2001). Cell-cycle profiles were analyzed with Watson Pragmatic algorithm in the FlowJo cytometry analysis software (Three Star, Inc., Ashland, OR).

Protein Secretion Assay
Protein secretion after bacterial infection was assayed as previously described (Collazo and Galán, 1997). Briefly, approximately 10\(^{5}\) cells were infected with wild-type S. Typhii or its isogenic \(\Delta\text{ptA} \text{ or } \Delta\text{ptB mutants, all expressing a } 3\times\text{FLAG-tagged cdTB, and 24 hr after infection, cells were detached from the plate by adding 30 μg/ml proteinase K and lysed in 0.3% Triton X-100 in PBS. Lysates were centrifuged 5 min at 20,000 × g, and the supernatants were filtered through a 0.2 μm filter. Filtered supernatants (representing the secreted fraction) and pellets (representing the bacterially associated fraction) were analyzed by western immunoblotting with an antibody directed to the FLAG tag.

Luciferase Reporter Assay
Henle-407 cells were infected with the reporter strain of S. Typhii as indicated above. At different times after infection, cells were washed and lysed in Passive Lysis Buffer (Promega Dual-Luciferase Reporter Assay System) and then sonicated. Firefly luciferase activity in lysates was measured using a luminometer as recommended by the manufacturer (Promega). Cells processed and infected in parallel in identical fashion were lysed with 0.1% deoxycholic acid, and dilutions of lysates were plated onto LB agar plates to determine the number of intracellular bacteria. Results were standardized based on the number of cfu.

Immunofluorescence Analysis
Infected cells were fixed in 4% paraformaldehyde, treated with 3% BSA, 4% goat serum, 0.3% Triton X-100, 50 mM NH\textsubscript{4}Cl in PBS, and labeled with the following primary antibodies: mouse monoclonal anti-FLAG M2 (Sigma; 1:10,000 dilution); rabbit polyclonal anti-Myc (Abcam; 1:2,000 dilution); and rabbit polyclonal anti-S. Typhii lipopolysaccharide (Difco Laboratories; 1:2,000 dilution), affinity-purified rabbit antibody raised against recombinant CdTB/pta/ptB complex (2 μg/ml). Secondary anti-rabbit and anti-mouse antibody conjugated to Alexa 488 or Alexa 594 were used at 1:2,000 dilution and 4’,6-diamidino-2-phenylindole (DAPI) was used at a concentration of 0.5 μg/ml. Images were acquired with an inverted microscope (Eclipse TE2000-U; Nikon) equipped with a CCD camera (MicroMAX RTE/CCD-1300Y; Princeton Instruments).

Protein Purification and Chromatography
Recombinant, polyhistidine-tagged pTa, pTb, or CdTB were expressed in E. coli, purified from inclusion bodies using nickel-affinity chromatography under denaturing conditions (8 M urea), and refolded together as previously described (Akeda and Galán, 2004). Nonsoluble materials were removed by centrifugation and filtration, and the resulting soluble proteins were used in the different biological and chromatographic assays. The PtA-PtB complex was purified using a Superdex 75 HR column (Amersham Pharmacia), equilibrated with 30 mM Tris-HCl pH 8.1, 1 mM DTT, and 5% glycerol, and run with a flow rate of 0.3 ml/min. To investigate the PtA/PtB/CdTB complex formation, the MonoQ column (Amersham Pharmacia) was loaded with the refolded complex (0.5 mg of each purified protein) (or CdTB alone) in a buffer containing 30 mM Tris-HCI pH 8.8, 1 mM DTT, and 5% glycerol. Proteins were eluted with 20 ml of a linear gradient of 0 to 500 mM NaCl in the same sample buffer at a flow rate of 1 ml/min.

Antibody Production and Affinity Purification
The PtA/PtB/CdTB complex was purified on a Superdex 200 HR column (Amersham Pharmacia) and used to immunize rabbits following standard procedures (Pocono Rabbit Farm and Laboratory, Canadensis, PA). For affinity purification, the PtA/PtB/CdTB complex was bound to a HitTrap NHS-activated HP column (GE Healthcare), and the antibody was purified according to the manufacturer’s instructions.

In Vitro ADP-Ribosylation Assay
Postnuclear supernatants from Henle-407 cells (50 μg) obtained by standard procedures were incubated with purified recombinant Pta (1 μg) in a buffer containing 30 mM Tris-HCl pH8, 1 mM DTT, and 50 μg/ml NAD\textsuperscript{3-P} in a final volume of 25 μl. The reaction mix was incubated for 1 hr at 37°C, stopped by heat inactivation for 15 min at 70°C, and trichloroacetic acid (TCA) was added to a final concentration of 10% in a final volume of 200 μl. The precipitation mix was incubated on ice for 1 hr, centrifuged, and washed three times with acetone. The pellet was dried at room temperature, resuspended in loading buffer, and submitted to SDS-PAGE. The gel was dried and exposed for autoradiography.

Supplemental Data
The Supplemental Data include eight supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/3/1/30/DC1/.

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