CdtC-Induced Processing of Membrane-Bound CdtA Is a Crucial Step in *Aggregatibacter actinomycetemcomitans* Cytolethal Distending Toxin Holotoxin Formation

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ABSTRACT *Aggregatibacter actinomycetemcomitans* is an oral pathogen causing periodontal disease and bacterial endocarditis. It produces cytolethal distending toxin (CDT) that could damage mammalian cells and tissues. CDT is a tripartite protein toxin composed of CdtA, CdtB, and CdtC. We have previously indicated that CdtA is a lipoprotein and that the proteolytic processing of CdtA is important for biogenesis and secretion of CDT holotoxin. Here, we established an *in vitro* processing assay of CdtA and investigated the interactions of CdtA with other Cdt subunits. This assay demonstrated that incubation of membrane-bound CdtA (MCdtA), CdtB, and CdtC immediately generated a processed form of CdtA (CdtA'), which is recovered from the soluble fraction. In contrast, incubation of soluble membrane-unbound CdtA with CdtB and CdtC did not yield any CdtA'. Furthermore, incubation of CdtC with MCdtA was enough to induce rapid processing of MCdtA, whereas CdtB alone was unable to induce the processing. Coimmunoprecipitation demonstrated that CdtA' and CdtC formed a complex. Furthermore, subsequent addition of CdtB to this reaction mixture resulted in complete CDT holotoxin complex. The cytolethal distending activity assay demonstrated that CDT complex containing CdtA' showed far stronger cytotoxicity than that containing CdtA. Collectively, our data suggest that CDT holotoxin formation *in vivo* is a sequential event: interaction of MCdtA and CdtC induces proteolytic processing of MCdtA, and the released CdtA' forms a complex with CdtC. Subsequent binding of CdtB to the CdtA'/CdtC complex results in CDT holotoxin formation.

KEYWORDS cytolethal distending toxin

Cytolethal distending toxin (CDT) is a bacterial exotoxin produced by several Gram-negative pathogenic bacteria, including *Escherichia coli* (1–3), *Campylobacter jejuni* (4, 5), *Haemophilus ducreyi* (6), *Shigella dysenteriae* (7), *Helicobacter hepaticus* (8, 9), *Helicobacter cinaedi* (10), and *Aggregatibacter actinomycetemcomitans* (11–13). CDT induces cell distension, cell cycle arrest, and death in mammalian host cells. *A. actinomycetemcomitans* CDT (AaCDT) has also been shown to induce G₂ cell arrest and/or apoptosis in various cell types (14–18). Moreover, CDTs that are produced by diverse human pathogens may contribute to the initiation and development of various diseases. Young et al. (19) provided evidence for the potential proinflammatory activity of the CDTs of *H. hepaticus* in a murine model of inflammatory bowel disease. We

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FIG 1 Distribution of CdtA and CdtA' forms in isolated bacterial fractions. The bacterial fractionation of *E. coli* carrying pQEcdtABC (A) or pQEcdtA (B) was performed after toxin expression by 1 mM IPTG. The whole-cell fraction (harvested cells) and culture supernatant were obtained by centrifugation, and membrane and periplasm fractions were obtained by ultracentrifugation after spheroplast isolation and by EDTA treatment under hypertonic conditions, as described in Materials and Methods. Each fraction was analyzed by SDS-PAGE and immunoblot analysis with rabbit anti-CdtA serum. For each panel, immunoblots were compiled from two separate images on two films of the same electrophoresis.

previously reported that AaCDT induced cell cycle arrest and damage in periodontal epithelial cells *in vivo* using a rat model (20). Our findings suggest that CDT-induced cell cycle arrest is one of the initiation pathways that result in the onset of periodontitis.

CDT is a tripartite AB toxin composed of three subunits, CdtA, CdtB, and CdtC (21). CdtB is the active (A) subunit, while CdtA and CdtC constitute the heterodimeric binding (B) subunit. CDT subunits are encoded on the *cdtA*, *cdtB*, and *cdtC* genes, which are located in tandem at the chromosomal *cdt* locus (3, 13, 22). The presence of three components, CdtA, CdtB, and CdtC, is necessary for the full toxicity of CDT holotoxin (23–26). CdtA and CdtC in a complex form a ricin-like domain, which is involved in the delivery of CdtB into host cells (24). CdtA and CdtC possess the two characterized structural elements of an aromatic patch and groove on the complex surface, which play an important role in binding to the cellular receptor (27). Thus, a clearer understanding of subunit interactions and the assembly of CDT holotoxin will provide insights into the biogenesis of CDT functions.

We previously demonstrated that *A. actinomycetemcomitans* CdtA with the lipid-binding consensus lipobox Leu-Val-Ala-Cys underwent lipid modifications at its N terminus and anchored in the outer membrane prior to extracellular secretion (28). Furthermore, the CdtA component of the complex in the culture supernatant was an N-terminally truncated form of CdtA (CdtA') of 18 to 19 kDa, whereas that in the periplasm was a 23-kDa form representing a CdtA with its signal sequence cleaved, suggesting that CdtA undergoes processing within bacterial cells, possibly in the periplasm (28). Lipid modifications in CdtA are important for the secretion of CDT holotoxin. Based on these findings, we proposed that CDT subunit assembly and the processing of CdtA occur in the periplasm for its secretion. However, the mechanisms underlying formation of a complex of CdtA (CdtA'), CdtB, and CdtC remain unclear. We here examined complex formation by these subunits using an *in vitro* processing assay and also investigated the mechanisms underlying the assembly of CDT holotoxin.

RESULTS

CdtA undergoes processing to form an N-terminal truncated CdtA in the periplasm. In order to clarify the significance of the N-terminal processing of CdtA in the process of complex formation, we examined the CdtA component in various fractions of *E. coli* M15 expressing only CdtA and in a fraction expressing all subunits, CdtA, CdtB, and CdtC, using Western blotting with anti-CdtA serum. In the whole-cell lysate of *E. coli* expressing CdtA, CdtB, and CdtC, a major component of CdtA was the unprocessed form, depicted as the CdtA form in Fig. 1A. Similarly, in the membrane fraction, more CdtA was present than CdtA'. On the other hand, a major component of CdtA in the periplasm and culture supernatant was the CdtA' form. The amount of the CdtA form was negligible in these fractions. These results clearly suggest that the processing of CdtA occurs in the periplasm/membrane and support the conclusion that

FIG 2 *In vitro* processing of membrane-bound and soluble CdtA. An *in vitro* processing assay was performed using membrane-bound CdtA (MCdtA) and membrane-unbound soluble CdtA (SCdtA) prepared from *E. coli* carrying pQEcdtA. MCdtA and SCdtA were incubated at 37°C with PBS. After incubation for the indicated times, the reaction mixtures were subjected to SDS-PAGE and immunoblotting using rabbit anti-CdtA serum.

the CdtA component in holotoxin in the culture supernatant is CdtA' (processed form) (28). When only CdtA was expressed in *E. coli*, the ratios of CdtA to CdtA' in the whole-cell lysate and membrane fractions were similar to those of the corresponding fractions of *E. coli* expressing CdtA, CdtB, and CdtC (Fig. 1B). However, a significant amount of the unprocessed form, CdtA, was present in the periplasm, and only the unprocessed form was found in the culture supernatant. These results suggest that other subunits of Cdt (i.e., CdtC and CdtB) are required for the secretion of CdtA'.

The interaction between MCdtA and CdtC promotes the formation of CdtA'. We focused on the processing of CdtA in the membrane fraction. We performed *in vitro* processing of membrane-bound CdtA (MCdtA) using a membrane preparation of *E. coli* expressing CdtA. The membrane preparation suspended in phosphate-buffered saline (PBS) was incubated at 37°C. When samples were analyzed by Western blotting, the unprocessed form of CdtA was predominant at time zero (Fig. 2A). After samples were incubated in PBS at 37°C, very slow processing from CdtA to CdtA' was observed (Fig. 2A). This processing did not occur when purified CdtA from the culture supernatant (SCdtA) was incubated in PBS (Fig. 2B). The addition of the periplasm fraction to the reaction mixture did not significantly improve the processing speed (data not shown). This result suggests that only MCdtA undergoes proteolytic processing even though the processing speed is very slow. We speculated that membrane anchoring may be important and that the protease component(s) associated with the membrane fraction may be involved in this event.

We then investigated whether other components of CDT affect this processing of CdtA. We incubated MCdtA with or without purified components of CdtB and CdtC. After incubation for the times indicated in Fig. 3, the membrane and soluble fractions were separated by ultracentrifugation, and soluble and pelleted fractions were immunodetected using anti-CdtA serum. When MCdtA was incubated alone in PBS at 30°C, the obvious processing of CdtA was not detected within 4 h, and CdtA remained in the pelleted fraction as the CdtA form (Fig. 3A). Purified CdtA from the culture supernatant (SCdtA) did not undergo processing when incubated in PBS at 30°C (Fig. 3B). When MCdtA was incubated with purified CdtB and CdtC, the immediate processing of CdtA to CdtA' just after the addition of CdtB and CdtC occurred, and processed CdtA' was immediately recovered from the supernatant fraction after ultracentrifugation (Fig. 3C). The unprocessed form (CdtA) and traces of CdtA' were noted in the membrane fraction. On the other hand, the combination of SCdtA with CdtB and CdtC did not induce the processing of CdtA (Fig. 3D). These results suggest that membrane-bound CdtA undergoes efficient processing in the presence of CdtB and CdtC and that the processed form CdtA' is released from the membrane to become a soluble form.

In order to clarify whether one or both of the components is needed to stimulate the processing of CdtA, MCdtA was incubated with either CdtB or CdtC, and the processing of CdtA was monitored. The results obtained revealed that only CdtC accelerated the processing of CdtA in the membrane fraction (Fig. 4A and B). There was no soluble form of CdtA' in the reaction mixture of MCdtA and purified CdtB (Fig. 4B). However, the further addition of CdtC immediately induced the processing of CdtA, and CdtA' was

FIG 3 *In vitro* processing of MCdtA in the presence of other CDT subunits. An *in vitro* processing assay was performed at 30°C using membrane-bound CdtA (MCdtA) (A), soluble CdtA (SCdtA) (B), MCdtA and purified CdtB and CdtC (C), and SCdtA and purified CdtB and CdtC (D). The reaction mixture containing MCdtA was separated into a supernatant (soluble fraction) and precipitant (membrane fraction) by ultracentrifugation and then subjected to SDS-PAGE following immunoblotting using rabbit anti-CdtA serum. Data in panel A represent the compilation of three images (MCdtA, soluble fraction, and membrane fraction) obtained from the same blot after electrophoresis. Data in panel C represent the compilation of two images (soluble fraction and membrane fraction) obtained from the same blot after electrophoresis.

recovered from the soluble fraction. These results suggest that the presence of CdtB did not stimulate processing of MCdtA and that CdtB did not interfere with the CdtC-induced processing of CdtA (Fig. 4B). As shown in Fig. 4C, the CdtC-induced processing of CdtA was CdtC dose dependent.

CdtC binding to membrane-bound CdtA leads to the facile processing of CdtA.

In order to establish whether CdtA' appeared in the supernatant fraction (soluble) as a complex of CdtA'/CdtC after the incubation of MCdtA with the CdtC present, we performed immunoprecipitation (IP) and Western blotting analyses. After the incuba-

FIG 4 *In vitro* processing of MCdtA with other sequentially added CDT components. An *in vitro* processing assay was performed using MCdtA and either purified CdtB or purified CdtC. (A) MCdtA was incubated with CdtC at 30°C. The lane "Add CdtB" represents the fraction in which 100 µl of MCdtA was incubated with 100 µl of CdtC for 30 min, and then 100 µl of CdtB was incubated further at 30°C for 5 min. (B) MCdtA was incubated with CdtB at 30°C. The lane "Add CdtC" represents the fraction in which MCdtA was incubated with CdtB for 30 min, and CdtC was incubated at 30°C for 5 min. (C) MCdtA was incubated with purified CdtC at different concentrations at 30°C for 30 min.

FIG 5 Identification of CDT subunits in complex by immunoprecipitation following a subunit incubation. (A) The soluble fraction and membrane fraction were subjected to ultracentrifugation after the incubation of MCdtA and CdtC. Closed and open arrowheads indicate CdtA and CdtA', respectively. Open arrows indicate CdtC. (B) The precleaned soluble and membrane fractions from the experiment shown in panel A were immunoprecipitated with anti-CdtA serum and detected with rabbit anti-CdtC serum. Open arrows indicate CdtC. (C) The soluble fraction and membrane fraction were subjected to ultracentrifugation after the incubation of MCdtA and CdtB. Closed and open arrowheads indicate CdtA and CdtA', respectively. Closed arrows indicate CdtB. (D) The precleaned soluble and membrane fractions from the experiment shown in panel C were immunoprecipitated with anti-CdtA serum and detected with rabbit anti-CdtB serum. Closed arrows indicate CdtB. (E) The soluble fraction was subjected to ultracentrifugation after the incubation of MCdtA, CdtB, and CdtC. Closed and open arrowheads indicate CdtA and CdtA', respectively. Closed and open arrows indicate CdtB and CdtC, respectively. (F) The precleaned soluble fraction from the experiment shown in panel E was immunoprecipitated with anti-CdtA serum and detected with rabbit anti-CdtB serum and rabbit anti-CdtC serum. Closed and open arrows indicate CdtB and CdtC, respectively. The asterisk indicates a nonspecific protein band. Imm-blot, immunoblot.

tion, the soluble fraction (supernatant) and membrane fraction (pellet) recovered by ultracentrifugation were immunoprecipitated using anti-CdtA serum and immunode-
 tected for CdtC. As shown in Fig. 5A, CdtC was detected mostly in the soluble fraction
 together with CdtA' before IP, while no CdtC was detected in the membrane fraction.
 Immunoprecipitation with anti-CdtA serum revealed that CdtC in the soluble fraction
 was predominantly detected in complex with CdtA' (Fig. 5A and B, IP beads). When
 MCdtA was incubated with CdtB, CdtB was also detected in the soluble fraction before
 IP (Fig. 5C). After IP, CdtB was detected unbound in the soluble fraction (Fig. 5D). These
 results suggest that the majority of CdtC is present as a complex of CdtA'/CdtC in the
 soluble fraction after interaction with MCdtA. In contrast, a CdtA/CdtB complex was

FIG 6 Cytolethal distending activity of the CDT complex. (A) The cytotoxic activities of various mixture patterns of CDT components were compared using HeLa cells. A mixture of CdtA', CdtB, and CdtC was prepared in two ways. The soluble fraction after the incubation of MCdtA and CdtC was incubated with purified CdtB. A mixture of CdtA, CdtB, and CdtC was prepared by incubating SCdtA from *E. coli* carrying pQEcdtA and purified CdtC and CdtB. Purified CdtA' was incubated with purified CdtC and CdtB. (B) CdtA or CdtA' in each constructed CDT complex was detected by SDS-PAGE and immunoblot analysis using rabbit anti-CdtA serum.

hardly found in IP beads following immunoprecipitation using the CdtA antibody (Fig. 5D). Most CdtB was present in the soluble fraction and did not bind to IP beads after immunoprecipitation, suggesting that CdtB does not directly or primarily bind to MCdtA.

CDT holotoxin formation through the binding of CdtA'/CdtC and CdtB. The above results clearly indicated that the incubation of MCdtA and CdtC immediately induced the processing of CdtA and release of CdtA' into the soluble fraction, in which CdtA' and CdtC form a complex. We previously demonstrated that *A. actinomycetemcomitans* holotoxin was composed of CdtA', CdtB, and CdtC in the culture supernatant of *A. actinomycetemcomitans* and *E. coli* carrying CdtABC (28). Therefore, we incubated the soluble fraction presumably containing CdtA' and CdtC as a complex with purified CdtB, and immunoprecipitation was performed using anti-CdtA serum in order to identify the components forming a complex with CdtA'. As shown in Fig. 5E, we detected CdtA', CdtB, and CdtC in the soluble fraction, as expected. Immunoprecipitation using anti-CdtA serum detected CdtB and CdtC in the IP bead-bound fraction (Fig. 5F). We then compared the toxic activities of various mixture patterns of CDT components. A mixture of CdtA', CdtB, and CdtC was prepared in two ways. MCdtA and CdtC were incubated, and the resulting supernatant fraction obtained by centrifugation was incubated with purified CdtB. CdtA' was also prepared as described in Materials and Methods and then incubated with CdtC, followed by CdtB. On the other hand, a mixture of SCdtA, CdtB, and CdtC was also similarly prepared. The presence of CdtA or CdtA' in these mixtures was confirmed by immunoblotting using anti-CdtA serum (Fig. 6B). As shown in Fig. 6A, the mixture consisting of CdtA', CdtC, and CdtB showed significantly stronger cytolethal distending activity than that of the mixture containing SCdtA, CdtC, and CdtB. Collectively, these results suggest that the presence of CdtA' enables the efficient formation of CDT holotoxin and that the processing of membrane-bound CdtA is an important step in the formation of highly active CDT holotoxin *in vivo*.

DISCUSSION

CDT is an exotoxin produced by several Gram-negative pathogenic bacteria, and its cytotoxic activity has been extensively characterized. However, few studies have attempted to elucidate the molecular mechanisms underlying the biogenesis and secretion of CDT holotoxin (28, 29). We previously demonstrated that *A. actinomycetem-comitans* CdtA has the lipid-binding consensus domain at the N-terminal signal sequence and also that lipid-modified CdtA is present in the outer membrane after posttranslational processing by the lipoprotein-specific signal peptidase II (28). Furthermore, since the N-terminally truncated CdtA form, CdtA', existed in the periplasm and culture supernatant, we suggested that lipid-modified CdtA underwent further processing of the N terminus (28). In the fractionation assay, we demonstrated that CdtA' was predominant in the periplasm of *E. coli* expressing CdtA, CdtB, and CdtC (Fig. 1A). This result clearly suggests that the N-terminal processing of CdtA occurs in the periplasm/membrane.

CdtA and CdtC serve as binding components to target cells and are necessary for the delivery of CdtB into host cells (27, 30). The formation of a heterotrimeric complex is indispensable for the virulence of CDT. We hypothesized that the formation of the CDT holotoxin complex occurs in the periplasm/membrane because CdtA', CdtB, and CdtC exist in the periplasm. The behavior of CdtA anchored to the outer membrane is important in CDT complex formation. We established an *in vitro* processing assay using the periplasm isolated from *E. coli* or CDT subunits in this study. The interaction between membrane-anchored CdtA and CdtC quickly generated CdtA' in the soluble fraction. Since CdtA' and CdtC were identified as a complex, the binding of CdtC to membrane-anchored CdtA was suggested to lead to CDT complex formation.

The enzyme(s) involved in the processing of MCdtA may be as yet an unidentified protease(s) present in the membrane or periplasm. The very slow processing of MCdtA incubated at 37°C and the observation that its addition to the periplasm fraction did not significantly stimulate this processing suggest that the processing enzyme may be associated with the membrane. In our *in vitro* processing assay, a certain amount of the CdtA form still remained in the membrane fraction after 4 h when MCdtA was incubated with CdtC. Although we do not have biochemical evidence for this, we speculate that the total amount of MCdtA in the membrane preparation is extremely high in *E. coli* carrying the multicopy CdtA expression plasmid, while the processing enzyme(s) is present in physiological amounts. Thus, only the portion of MCdtA that is associated with the processing protease is cleaved upon interaction with CdtC. This speculation needs further verification after the enzyme(s) involved in this process has been identified.

Previous studies on CDT complex formation were exclusively performed using recombinant purified toxin components (26, 31, 32). The findings obtained clearly suggested that the AaCDT subunits, CdtA, CdtB, and CdtC, easily reconstitute the CdtABC complex and exhibit CDT holotoxin activity (30). Hu and Stebbins (32) investigated the dynamics and assembly of CDT holotoxin and subunit interactions. They demonstrated that the estimated binding affinities of CdtA and CdtC were markedly higher than the affinity of CdtB bound with CdtA or CdtC. Additionally, Shenker et al. (26) examined the relationships among three CDT subunits from *A. actinomycetem-comitans*. Their findings suggested that the assembly of the stable holotoxin complex requires CdtA and CdtC and that CdtA and CdtC must form a complex before CdtB is able to associate with the holotoxin. Although their conclusion may be accurate, they did not explain why CdtA and CdtC had markedly higher affinities. Our results suggest a favorable reason: three components may not coexist as soluble CdtA, CdtB, and CdtC to interact with each other in the periplasmic space. Instead, a cascade reaction of the CdtC-induced processing of MCdtA with the concomitant generation of the CdtA'/CdtC complex and subsequent formation of a trimeric complex with CdtB may result in the rapid and efficient formation of holotoxin *in vivo*. In support of this, the CdtA'/CdtB/CdtC complex was previously reported to be present in the culture supernatant of *E.*

FIG 7 Model of CDT complex formation and secretion. Immature CdtA, CdtB, and CdtC are synthesized in the cytoplasm and secreted into the periplasmic compartment in a *sec*-dependent general secretion pathway using their N-terminal signal sequences. During passage through the inner membrane, the signal sequences of immature CdtB and immature CdtC are cleaved by signal peptidase I, and CdtB and CdtC become mature forms. Unmodified CdtA is cleaved at its signal sequence by signal peptidase II and modified with lipid. Lipid-modified CdtA (lipoCdtA), using the serine residue next to a lipid-modified cysteine residue, is carried to the outer membrane. This membrane-bound CdtA preferentially forms a complex with CdtC in the outer membrane, and the N-terminally truncated processing of membrane-bound CdtA is promoted by CdtC binding. Subsequently, the complex of CdtA' and CdtC is released into the periplasmic space. Finally, CDT holotoxin is formed in the periplasm and secreted into the culture medium.

coli expressing CdtABC (28). Furthermore, the CdtA'/CdtB/CdtC complex exhibited significantly higher CDT activity than the SCdtA/CdtB/CdtC complex. Our results strongly suggest that the processing of membrane-bound CdtA and subsequent formation of the CdtA'/CdtB/CdtC complex are important processes in the maturation of CDT holotoxin.

Figure 7 shows our current model for the biogenesis of *A. actinomycetemcomitans* CDT holotoxin based on the present results and previous findings (28). We suggest that an unmodified CdtA, immature CdtB, and immature CdtC are secreted into the periplasmic space through a *sec*-dependent secretion pathway. After translocation to the inner membrane, immature CdtB and CdtC undergo processing by signal peptidase I. Unmodified CdtA undergoes processing by lipoprotein-specific signal peptidase II, followed by lipid modifications. Lipid-modified CdtA is transported to the outer membrane by the Lol system, which transports lipoproteins in the periplasm to the outer membrane (33) following amide-linked fatty acid modifications at the N-terminal cysteine. After membrane-bound lipid-modified CdtA interacts with CdtC, the processing of CdtA immediately occurs, and the complex of CdtA' and CdtC is formed and subsequently released into the periplasm. The CdtA'-CdtC complex and CdtB then form a complex in the periplasm. Holotoxin may be released from the cell body through an as yet unidentified pathway or by outer membrane vesicles (OMVs) (34).

The possible lipobox consensus sequence of CdtA may be found in a number of bacteria, including *Aggregatibacter*, *H. ducreyi*, *C. jejuni*, *H. hepatica*, and *E. coli* (28). However, *E. coli* also carries the consensus sequence in CdtC. Therefore, it currently remains unclear whether this picture is applicable to the genesis of CDT holotoxin in *E. coli*. Our results suggest that a mature *A. actinomycetemcomitans* CDT complex is constructed in the periplasm and then secreted extracellularly, and they provide a better understanding of the mechanisms underlying processing and subunit assembly

TABLE 1 Strains used in this study

<i>E. coli</i> strain and plasmid	Description (restriction enzymes)	Source or reference
M15		
pQEcdtABC	cdtABC on pQE60 (NcoI-BglII)	28
pQEcdtA	cdtA on pQE60 (NcoI-BglII)	This study
pQEcdtC	cdtC on pQE60 (NcoI-BglII)	This study
DH5 α		
pQEcdtB	cdtB on pQE60 (NcoI-BglII)	This study

in the periplasm compartment during the biogenesis of CDT holotoxin. Further studies on the release mechanisms of CDT holotoxin are needed in order to obtain a deeper understanding of the overall secretion mechanisms of CDT.

MATERIALS AND METHODS

Bacterial strains and growth medium. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* was grown aerobically in lysogeny broth (LB) (5 g of yeast extract, 10 g of Trypticase peptone, and 10 g of NaCl per liter, pH 7.2) or on LB agar plates. Ampicillin (50 μ g/ml) was added when necessary.

Preparation of crude fractions. CDT holotoxin and the CdtA subunit were obtained from *E. coli* carrying pQEcdtABC (15) and pQEcdtA, respectively. CDT or CdtA expression in *E. coli* was induced by the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 4 h at an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.7. Several crude CDT fractions were prepared from a 500-ml culture of *E. coli* carrying pQEcdtABC or pQEcdtA as follows. For the whole-cell fraction, harvested cells were washed with phosphate-buffered saline (PBS) 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄ at pH 7.0 and resuspended in PBS at an approximately 10-fold volume of the wet cell pellet. Cells in suspension were disrupted using an ultrasonic disruptor (UD-200; Tomy, Tokyo, Japan) for 30 s, five times at an output level of 4, on ice. Unbroken cells were removed by centrifugation at 5,000 $\times$ g at 4°C for 10 min, and the supernatant was used as the whole-cell fraction. An approximately 10-fold dilution of the whole-cell fraction was applied to SDS-PAGE gels. For the membrane and periplasm fractions, a modification of the method described by Osborn et al. (35) was used for the preparation of samples. Briefly, recombinant *E. coli* cells were harvested at an OD₆₀₀ of 0.9 after induction with 1 mM IPTG for 4 h. Harvested cells were resuspended in 9 ml of 0.75 M sucrose and 10 mM Tris-HCl at pH 7.8. The cell wall peptidoglycan was digested with 90 μ l of 10 mg/ml lysozyme (Sigma) for 2 min on ice. Spheroplasts were prepared by gradually adding 18 ml of 1.5 mM EDTA. The spheroplasts were ultrasonically disrupted (UD-200; Tomy) for membrane preparation. Unbroken cells were removed by centrifugation at 5,000 $\times$ g for 10 min. The membrane fraction was prepared from the supernatant by ultracentrifugation (Optima TL Ultracentrifuge; Beckman, CA, USA) at 100,000 $\times$ g at 4°C for 50 min as the precipitate. The precipitate of ultracentrifugation was suspended in 100 μ l of 10 mM PBS as the membrane preparation. For periplasm preparation, harvested cells were suspended in 10 ml of 0.2 M Tris-HCl (pH 8.0) containing 1 M sucrose, 1 mM EDTA (pH 8.0), 0.5 mM benzamidine, and 0.02 mg/ml soybean trypsin inhibitor and gently mixed for 1 h on ice. The suspension was centrifuged twice at 9,700 $\times$ g at 4°C for 20 min. The supernatant was concentrated 50-fold using Amicon Ultra 0.5-ml centrifugal filters (Merck Millipore, MA, USA) and used as the periplasm preparation. An appropriate dilution of the membrane fraction was applied to SDS-PAGE gels. The membrane fraction of *E. coli* carrying pQEcdtA was used as membrane-bound CdtA (MCdtA). For the supernatant fraction, the culture in which cells reached the stationary phase was centrifuged at 10,000 $\times$ g at 4°C for 15 min. A concentrated culture supernatant was prepared using an 80% saturated ammonium sulfate precipitation of the culture supernatant, followed by dialysis with 10 mM phosphate-buffered saline (PBS; pH 7.0) (13). The dialysate was concentrated to ca. 10 times the volume using an Amicon Ultra 10K concentrator (Merck Millipore). CdtA from the supernatant fraction of *E. coli* carrying pQEcdtA was used as membrane-unbound soluble CdtA (SCdtA).

Protein assay. The protein concentration of each fraction was measured at the OD₅₉₅ using a Bio-Rad protein assay kit. Ten microliters of each fraction was diluted in 800 μ l with H₂O and mixed with 200 μ l of the dye reagent provided in the kit. After incubation at room temperature for 5 min, absorbance was measured at 595 nm using an Ultrospec 100 Pro instrument (GE Healthcare, CT, USA).

Purification of subunits CdtB and CdtC. CdtB and CdtC were prepared from *E. coli* carrying pQEcdtB and pQEcdtC, respectively, as C-terminal 6 $\times$ His-tagged proteins. The cell lysate was prepared as described above from harvested cells after induction with 1 mM IPTG for 4 h. A total of 1 ml of Ni-chelated agarose beads (Qiagen) was added to the lysate, and the mixture was gently shaken at 4°C overnight. The beads were recovered by an open column and washed with 40 ml of cold wash buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 20 mM imidazole), and protein was then eluted with 5 ml of elution buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 400 mM imidazole). The eluted fraction was used as a CDT subunit preparation after dialysis against 10 mM PBS and concentrated to ca. 10 times the volume using an Amicon Ultra 10K concentrator (Merck Millipore).

In vitro processing assay of CdtA. MCdtA or SCdtA was used as a substrate for the *in vitro* processing of CdtA. One hundred microliters of MCdtA or SCdtA (1 mg/ml total protein) was incubated with 100 μ l of 10 mM PBS or the periplasmic fraction of strain M15 at 37°C. The reaction mixture was

immediately stored on ice and subjected to SDS-PAGE followed by Western blotting using anti-CdtA serum, as described elsewhere (13).

In vitro processing assay of CdtA in the presence of other Cdt components. Since a preliminary experiment suggested that the incubation of MCdtA alone at 30°C did not induce the processing of CdtA, we employed 30°C for this assay. MCdtA or SCdtA was used as the substrate for *in vitro* processing. One hundred microliters of MCdtA or SCdtA (1 mg/ml protein) was incubated with 100 μ l each of CdtB and CdtC (100 μ g/ml) at 30°C for various periods. In order to investigate the effects of the interaction between the two subunits, 100 μ l of the mixture of MCdtA, 100 μ l of CdtB or CdtC in 10 mM PBS, and 100 μ l of 10 mM PBS were incubated at 30°C. After the incubation, the reaction mixture was immediately transferred onto ice and separated into soluble and membrane fractions by ultracentrifugation at $100,000 \times g$ at 4°C for 50 min. In order to test various patterns of the mixture of Cdt components, the mixing of Cdt components was performed in two steps: a first step consisting of mixing a fraction of 100 μ l MCdtA and 100 μ l CdtC or CdtB and a second step consisting of an additional incubation with 100 μ l of the CdtB for the mixture of MCdtA and CdtC or 100 μ l of the CdtC for the mixture of MCdtA and CdtB.

Coimmunoprecipitation. After the *in vitro* processing assay, samples were used for a pulldown assay. The supernatant (soluble fraction) and appropriate dilution prepared from the precipitation (membrane fraction) of the CDT component mixture after ultracentrifugation were precleaned with 20 μ l of protein G-Sepharose 4 beads (Amersham), and beads were removed by centrifugation at $10,000 \times g$. The supernatant was incubated with 0.5 μ l of rabbit anti-CdtA serum (13) at 4°C for 1 h. Twenty microliters of protein G-Sepharose 4 beads was then added and incubated further at 4°C for 1 h. The beads were pelleted by centrifugation at $5,000 \times g$ for 5 min, washed three times in 50 mM Tris-buffered saline (pH 7.5) supplemented with 0.05% Tween 20, resuspended in 30 μ l of SDS sample buffer containing 2% SDS, 10% β -mercaptoethanol, 0.01% bromophenol blue, 20% glycerol, and 120 mM Tris-HCl (pH 6.8), and treated at 95°C for 5 min. The beads were pelleted again by centrifugation, and the supernatant was analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

HeLa cell culture. HeLa cells (ATCC CCL2) were cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂–95% air atmosphere.

Assay for CDT activity. CDT activity was measured for various patterns of the CDT complex produced by the mixing of CDT components containing MCdtA or SCdtA (as described above). The CDT complex containing CdtA', which was produced from a 24-h incubation of MCdtA and the periplasmic fraction of strain M15 by an *in vitro* processing assay, was prepared as the control. HeLa cells were plated on a 96-well plate (Falcon; Becton Dickinson) at a concentration of 2×10^3 cells per 100- μ l well 1 day before the experiment. A total of 100 μ l of the mixture of CDT components *in vitro* was inoculated into the HeLa cell monolayer in 100 μ l of medium in the first well of the 96-well plate. After the sample and medium were mixed well, a half- μ l (100 μ l) of the sample-medium mixture was added to the next well, and a 2-fold dilution was serially performed. Morphological changes were observed using phase-contrast microscopy (Diaphot 300; Nikon, Tokyo, Japan) at day 2. Cell distension was defined as a more than 5-fold expansion in size from that of the control cells. Cytolethal distending activity was titrated using the endpoint as the highest 2-fold dilution of toxic material giving 50% transformed cells (50% cytotoxic concentration, CD₅₀) after a 48-h incubation.

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