Production of the polyclonal subunit C protein antibody against *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin

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ABSTRACT

Purpose: Cytolethal distending toxin (CDT) considered as a key factor of localized aggressive periodontitis, endocarditis, meningitis, and osteomyelitis is composed of five open reading frames (ORFs). Among of them, the individual role of CdtA and CdtC is not clear; several reports presents that CDT is an AB2 toxin and they enters the host cell via clathrin-coated pits or through the interaction with GM3 ganglioside. So, CdtA, CdtC, or both seem to be required for the delivery of the CdtB protein into the host cell. Moreover, recombinant CDT was suggested as good vaccine material and antibody against CDT can be used for neutralization or for a detection kit.

Materials and Methods: We constructed the pET28a-cdtC plasmid from *Aggregatibacter actinomycetemcomitans* Y4 by genomic DNA PCR and expressed in BL21 (DE3) *Escherichia coli* system. We obtained the antibody against the recombinant CdtC in mice system. Using the anti-CdtC antibody, we test the native CdtC detection by ELISA and Western Blotting and confirm the expression time of native CdtC protein during the growth phase of *A. actinomycetemcomitans*.

Results: In this study we reconstructed CdtC subunit of *A. actinomycetemcomitans* Y4 and generated the anti CdtC antibody against recombinant CdtC subunit expressed in *E. coli* system. Our anti CdtC antibody can be interacting with recombinant CdtC and native CDT in ELISA and Western system. Also, CDT holotoxin existed at 24h but not at 48h meaning that CDT holotoxin was assembled at specific time during the bacterial growth.

Conclusion: In conclusion, we thought that our anti CdtC antibody could be used mucosal adjuvant or detection kit development, because it could interact with native CDT holotoxin. ***(J Korean Acad Periodontol 2008;38:335-342)***

KEY WORDS: *Aggregatibacter actinomycetemcomitans*; cytolethal distending toxin.

Introduction

Cytolethal distending toxin (CDT) was first reported as a toxin in *Escherichia coli* by Johnson and Lior[1]. The new toxin was designated as 'cytolethal distending toxin' to reflect the morphologic change associated with its activity on host cell. Also, CDT has a unique mechanism of host cell DNA damage, leading to cell cycle arrest inducing apoptosis[2]. To date, CDTs of various gram-negative bacterial species such as *Campylobacter Jejuni, Aggregatibacter actinomycetemcomitans*, and *Haemophilus ducreyi* are possible virulence factors, because of their cytolethal effect and associations with disease such as enteritis, hepatitis, gastroenteritis, chancroids, localized aggressive periodontitis, endocarditis, meningitis, and osteomyelitis[3-5].

A. actinomycetemcomitans is the only oral bacterium identified to produce a CDT[6,7]. The CDT gene of *A. actinomycetemcomitans* is composed of five open reading frames (ORFs): orf1, orf2, cdtA, cdtB, and cdtC[8]. The orf1 and orf2 located upstream of the cdtA gene are probably transcribed together with the three cdt genes. Still, it is not known whether orf1 and orf2 are actually translated in vivo. Although the individual role of CdtA and CdtC is not clear, several reports presents that CDT is an AB2 toxin (CdtB, the enzymatically active subunit A; CdtA and CdtC, the heterodimeric subunit B)[9]. Moreover, previous studies showed that CDT enters the host cell via clathrin-coated pits

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or through the interaction with GM3 ganglioside[10]. So, many other research groups suggest that CdtA, CdtC, or both seem to be required for the delivery of the CdtB protein into the host cell and also contribute to the cell cycle arrest[3,11-14]. Frisk et al.[15] suggest that CdtA and/or CdtC are the components of the holotoxin which anchor to the cell surfaces or act as a carrier for CdtB to internalize this subunit into the cells and to elicit its cellular toxicity. Cope et al.[16] proved that a monoclonal antibody against CdtC was able to hamper the toxic activity of CDT of *H. ducreyi*.

Recombinant CDT was good vaccine material and antibody against CDT can be used for neutralization or for a detection kit. More interestingly, there is a possibility of using CDT as a mucosal adjuvant through its binding activity to cell surface receptor[17]. Up to now, cholera toxin or enterotoxic *E. coli* toxin has been mainly utilized as a mucosal adjuvant but these toxins belong to AB5 family, while CDT to AB2. Development of a new mucosal adjuvant will be more feasible by using CDT due to its structural simplicity or lesser toxicity.

Expression of toxins is tightly controlled during the growth phase of pathogens and associates with the virulent activity of toxins. For example, Apx protein of A*ctinobacillus pleuropneumoniae* has hemolytic activity and was differentially expressed through the growth curve[18]. Similarly, the information about the expression of CDT or its secretion will be helpful to analyze the pathogenesis of *A. actinomycetemcomitans*.

Therefore, the goal of this study was to generate the polyclonal antibody with high affinity against recombinant CdtC. The anti-CdtC antibody was then utilized to detect native CdtC protein and to analyze the CDT expression pattern during the growth phase of *A. actinomycetemcomitans*.

Materials and Methods

1. Bacterial strains and culture conditions

A. actinomycetemcomitans Y4 (ATCC 43718) was cultured in Brain heart infusion (BHI) broth at 37°C in anaerobic chamber containing 85% N_2, 10% H_2, and 5% CO_2[5,19]. Growth was monitored by optical density at 600nm, with all batch cultures having an initial O.D. 600nm of approximately 0.01 after sub-culturing from overnight growth. For the expression of recombinant CdtC subunit, *E. coli* strain, BL21 (DE3), was cultured in Luria-Bertari (LB) medium at 37°C with shaking at 200rpm[7].

2. Expression and Purification of recombinant CdtC

Each colony of *E. coli* BL21 (DE3) transferred with the pET28a-cdtC plasmid was cultured into 5ml LB broth containing 50ug/ml kanamycine and incubated at 37°C for overnight with shaking at 200rpm. When all batch cultures have an initial O.D. 600nm of approximately 0.5, they were transferred into the 500ml LB broth containing 50ug/ml kanamycine and grown at 37°C with vigorously shaking until an optical density of 0.8 was reached. For expression of recombinant CdtC subunit, isopropyl-b-D-thiogalactopyranoside (IPTG) was added to 1 mM and further cultured for 3h at 30°C. Induced bacteria were harvested using centrifugation, and then the pellet was resuspended in 20mM Tris-HCl containing 0.5M NaCl, and 5mM imidazole (pH 8.0). The resuspended bacteria were disrupted with an ultrasonic disruptor[9,20]. After removing the soluble protein by centrifugation, the pellet was washed with Phosphate-buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, and 1.5mM KH2PO4, pH 7.3) and resuspended in Binding buffer (100 mM NaH_2PO_4, 10mM Tris HCl (pH 8.0), and 8M Urea). The solubilized solution was then loaded into the nickel-chelated agarose (Ni-NTA, Qiagen). The column was washed with Binding buffer and eluted with a solution of 100 mM NaH_2PO_4, 10mM Tris HCl (pH 4.5), and 8M Urea. Quantification of the purified proteins was carried out by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electro-

phoresis (SDS-PAGE) using known quantities of bovine serum albumin as standards.

3. Production of polyclonal anti-CdtC antibody

To generate anti-CdtC serum, BALB/c mice were immunized at day 1 with CdtC emulsified with Freund complete adjuvant and 2 weeks later with CdtC emulsified with Freund incomplete adjuvant. Antisera were obtained 5 weeks after first injection.

4. SDS-PAGE and Western blot

Bacterial lysate or purified recombinant CdtC was resolved by SDS-PAGE and transferred to nitrocellulose paper for Western blot analysis with polyclonal anti-CdtC antibody as previously described[7]. The transferred nitrocellulose membrane was blocked by 5% skim milk at room temperature for 1 h and then incubated with polyclonal anti-CdtC antibody (1:100 or 1:1000 dilution) for another hour. After the nitrocellulose membrane was incubated with anti-mouse IgG conjugated to horseradish peroxidase for 1h and then developed into the film using chemiluminescence (ECL) for 3min.

5. ELISA (Enzyme-Linked ImmunoSorbent Assay)

Recombinant CDT subunit protein or culture supernatant of *A. actinomycetemcomitans* Y4 resuspended in carbonate-bicarbonate buffer were used to coat ELISA plate at 37°C for 2 h and the coated plates was blocked by 1% skim milk. After washed using PBS (pH 7.5), the plates were incubated with polyclonal anti-CdtC antibody (1:100 or 1:1000) in PBS at 37°C for 2h. The plates were then washed with PBS, and further incubated with anti-mouse IgG conjugated with alkaline phosphatase in PBS (1:5000 dilutions) at 37°C for 2h and then applied with the substrate which is converted by the enzyme to elicit a chromogenic signal. The result was quantified using a spectrophotometer at O.D. 405nm.

Results

1. Production of polyclonal anti-CdtC antibody

A. actinomycetemcomitans belonging to the family *Pasteurellaceae* was involved in the pathogenesis of localized aggressive periodontitis. Among the toxins of *A. actinomycetemcomitans*, CDT was considered as an important virulent factor. Although CdtB has an enzyme activity eliciting cell cycle arrest, CdtC was associated with host cell binding and invasion[9,21]. To study CdtC function in detail, polyclonal anti-CdtC antibody was produced using recombinant CdtC protein.

Recombinant CdtC protein was expressed in an *E. coli* strain, BL21 (DE3), harboring pET28a-cdtC plasmid and purified by Ni-NTA agarose column. BLAB/C mice were first injected with purified recombinant CdtC resuspended with Freund complete adjuvant (100ug of CdtC per 1ml). After 2 weeks, mice were immunized by purified recombinant CdtC resuspended with Freund incomplete adjuvant (100ug of CdtC per 1ml) every week 5 times[22].

To obtain the anti CdtC sera, we bleed the immunized mice at 3 and 5 week. We tested whether these anti-CdtC sera may contain the specific antibody against recombinant CdtC subunit using Western blotting. The drawn antiserum clearly recognized recombinant CdtC protein (Fig. 1). Moreover, the antiserum reacted also to a native CdtC (17.4 kDa) in bacterial lysate of *A. actinomycetemcomitans* Y4 but not to the non-related protein, recombinant AgI/II fragment of Streptococcus mutans (Fig. 2). These results suggested that the serum contain the anti-CdtC antibody binding to the recombinant CdtC protein and native CdtC as well.

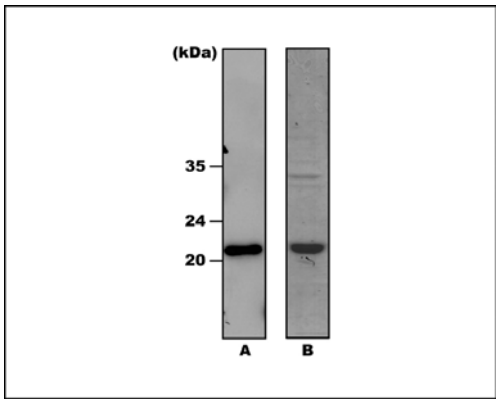

Figure 1. *SDS-PAGE analysis and Western blot of recombinant CdtC. Expression of recombinant CdtC protein was revealed by Western blotting using polyclonal anti-CdtC serum drawn at 5 weeks after first immunization (Lane A). Recombinant CdtC protein was purified by Ni-NTA column and shown on a SDS-PAGE gel (Lane B).*

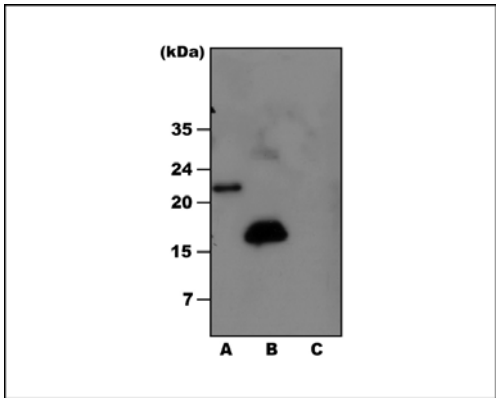

Figure 2. *Western blot of recombinant or native CdtC subunit. Recombinant CdtC (100ng, Lane A), the bacterial lysate of A. actinomycetemcomitans Y4 (100ng, Lane B) and recombinant AgI/II (100ng, Lane C) were analyzed by Western blotting using polyclonal anti-CdtC serum, respectively.*

2. ELISA condition for anti-CdtC antibody

ELISA condition for anti-CdtC sera was tested to detect native CDT protein[23]. ELISA plate coated by recombinant CdtC protein (100ng) was incubated with antiserum 1:100 or 1:1000 for 2h. After incubation of anti mouse IgG-AP and then AP substrate, the color intensity was monitored at 405nm every 10, 30, 60, 90, and 100min using spectrophotometer. When color density was compared, anti-CdtC polyclonal antibody (1:100 dilutions) distinguished the recombinant CdtC protein (100ng) since 10min and these results were confirmed serial dilution of anti-CdtC antibody (Fig. 3). At a higher dilution (1:1000), anti-CdtC polyclonal antibody also distinguished the recombinant CdtC protein (100ng) since 30min (Fig. 4). These data were converted in reciprocal Log2 titer and the reaction pattern and sensitivity of anti-CdtC polyclonal antibody compared (Fig. 5).

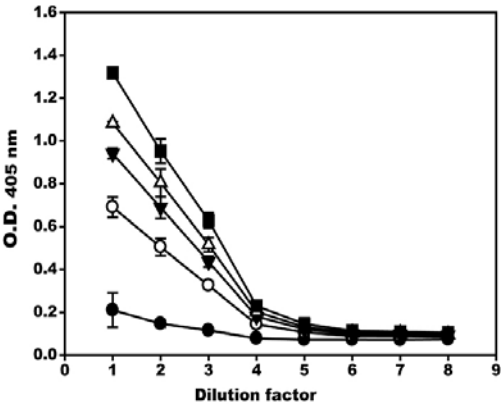

Figure 3. *ELISA using high concentration of anti-CdtC antibody. The recombinant CdtC protein (100ng) was detected by anti-CdtC antibody at 1:100 dilutions for diverse time points, 10min (closed circle), 30min (open circle), 60min (closed triangle), 90min (open triangle), and 100min (closed square).*

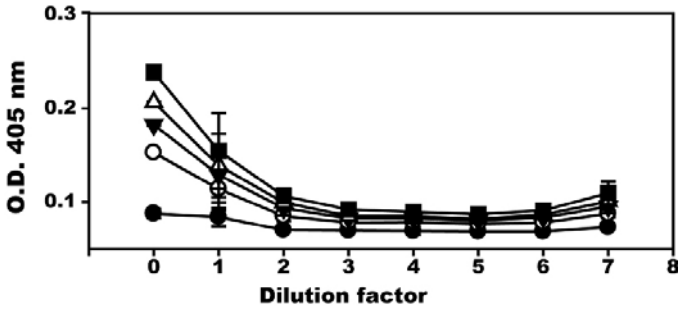

Figure 4. *ELISA using low concentration of anti-CdtC antibody. The recombinant CdtC protein (100ng) was detected by anti-CdtC antibody at 1:1000 dilutions for diverse time points, 10min (closed circle), 30min (open circle), 60min (closed triangle), 90min (open triangle), and 100min (closed square).*

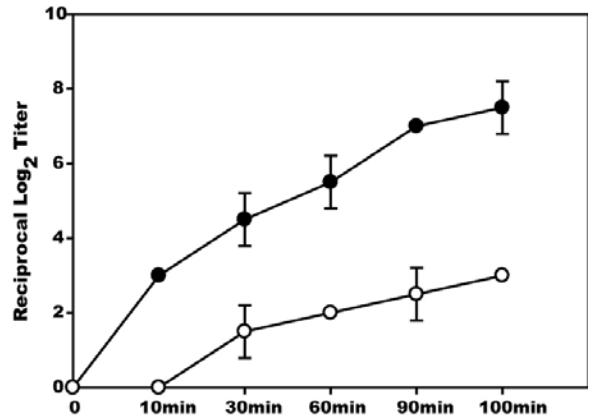

Figure 5. *Comparison of ELISA for low or high concentration of anti-CdtC antibody. The recombinant CdtC protein (100ng) was detected by anti-CdtC antibody at 1:100 (closed circle) and 1:1000 (open circle) dilutions for diverse time points (10 - 100min). Mean antibody titers are given as -log2 dilution.*

The bacterial culture supernatant containing native CdtC subunit or holotoxin CDT was used to coat in plate and then incubated with anti-CdtC polyclonal antibody (1:100 dilutions). The result of bacterial culture supernatant was shown the 60% than that of recombinant CdtC protein (Fig. 6). Also, to confirm the anti-CdtC polyclonal antibody specificity about CdtC subunit, ELISA plate was coated with the recombinant CdtA or CdtB and monitored at every 10, 30, 60, 90, and 100 min by spectrophotometer. Anti-CdtC polyclonal antibody (1:100 dilutions) only reacts with recombinant CdtC protein. Above results suggested that anti-CdtC polyclonal antibody was specific against CdtC protein, but not against CdtA or CdtB (Fig. 7).

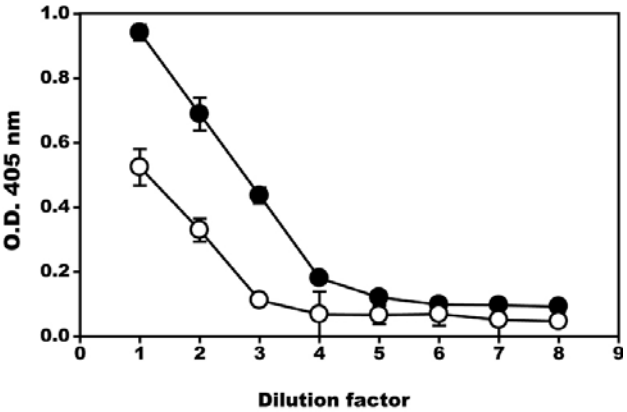

Figure 6. *Comparison of ELISA for recombinant CdtC or the culture supernatant of A. actinomycetemcomitans. The recombinant CdtC (100ng), (closed circle) or the culture supernatant of A. actinomycetemcomitans (open circle) was detected by anti-CdtC antibody at 1:100 dilutions for 60min.*

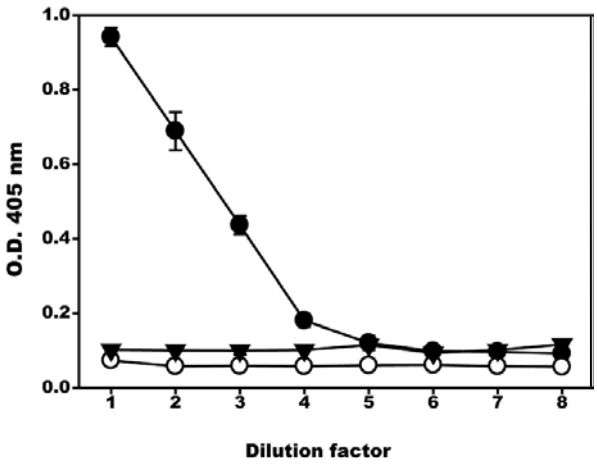

Figure 7. *Comparison of ELISA for recombinant CdtA, B, or C protein. The 100ng of recombinant CdtA (closed triangle), CdtB (open circle), or CdtC (closed circle) was detected by anti-CdtC antibody at 1:100 dilution for 60min.*

3. Expression of CdtC protein during the growth phase of *A. actinomycetemcomitans*

Through the Western blotting and ELISA using anti-CdtC polyclonal antibody, we expect that anti-CdtC polyclonal antibody could detect the native CdtC and CDT holotoxin. Therefore, anti-CdtC antibody will reveal the expression condition of CdtC protein in *A. actinomycetemcomitans* growth.

Firstly, we monitored the growth rate of *A. actinomycetemcomitans* Y4 by optical density at 600nm, with all batch cultures having an initial O.D. 600nm of approximately 0.01 after sub-culturing from over night growth (Fig. 8A)[17,22]. The culture supernatants and cell lysate were prepared (6, 9, 24, and 30h) and then analyzed using Western blotting. Although anti-CdtC polyclonal antibody had not detected the native CdtC subunit in the culture supernatant of *A. actinomycetemcomitans* Y4, native CdtC subunit was increased in a time-dependent way in cell lysate (Fig. 8B). Also, the protein band of holotoxin size was detected at 24 h cell lysate sample (Fig. 8C). This result implied that native CdtC were piled up on the cell membrane or periplasm and holotoxin assembly were maximized around late-log phase (Fig. 8).

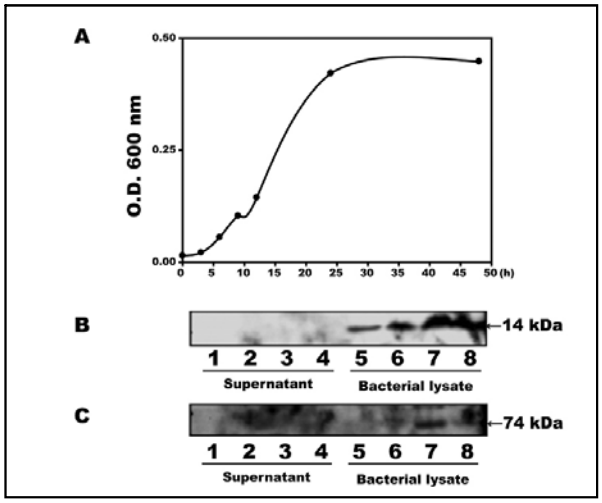

Figure 8. *CdtC subunit and CDT holotoxin production during A. actinomycetemcomitans growth. It is Growth rate of A. actinomycetemcomitans Y4 (A). Culture supernatant (Lane 1-4) or bacterial lysate (Lane 5-8) ot A. actinomycetemcomitans Y4 was prepared at 6, 9, 24, and 30h. CdtC subunit (B) was detected at 14 kDa and CDT holotoxin (C) was detected at 74kDa. All samples were separated on SDS-PAGE gel and transferred to nitrocellulose membrane followed by development using anti-CdtC antibody.*

Discussion

Aggregatibacter actinomycetemcomitans was associated with localized aggressive periodontitis, endocarditis, meningitis, and osteomyelitis. The CDT was considered as a key factor of these diseases[3,11-12,24]. Also, recent studies have shown that *A. actinomycetemcomitans* CDT inhibit human gingival fibroblasts, Chinese hamster ovary, monkey fibroblasts (cos1), human cervical carcinoma (HeLa), human laryngeal carcinoma (HEp2), murin B-cell hybridoma, human periodontal ligament cells, JY B-lymphoblastoid, human oral epidermoid carcinoma (KB), and primary T cells in the G2 phase of the cell cycle[6,25]. Moreover, this cytotoxic factor of *A. actinomycetemcomitans* has related with CDT of *Escherichia coli*, *Haemophilus ducreyi*, *Campylobacter Jejuni*, and *Helicobacter hepaticus*[16,26].

The specificity of polyclonal anti-CdtC antibody was proved. First, the anti-CdtC antibody reacted to recombinant or native CdtC subunit but not to recombinant AgI/II protein in Western blotting (Figs. 1 and 2). In ELISA assay, the antibody responded to recombinant CdtC in a dose-dependent way, but not to recombinant CdtA or CdtB proteins (Figs. 3-5 and 7). Another ELISA also revealed that the anti-CdtC antibody detects native CdtC protein from the bacterial culture supernatant (Fig. 6). For unknown reason, CdtC subunit from around log phase sample was better detected in ELISA than in Western blotting.

An enzymatic subunit of the CDT, CdtB has been known to be internalized into the host cell in order to induce its genotoxic effect. However, CdtB cannot be localized in host cytoplasm without the help of a heterodimeric complex consisting of CdtA and CdtC. So, some studies suggested that CdtC functions as a ligand to interact with GM3 ganglioside of host cell surface[10,21]. Other toxin such as Apx of *Actinobacillus pleuropneumoniae* has been known to be expressed at specific phase of growth[18]. Similarly, this study proved that the expression of CdtC subunit was regulated according to the bacterial growth phase. CdtC protein of the bacterial lysates started to show up in early log phase and its expression was maximized in late log phase, while CdtC expression in the culture supernatant was not clear. Interestingly, a protein band at the higher molecular weight than CdtC subunit existed for a short time around late log phase. These results implied that CDT expression is tightly controlled during the growth phase and CDT can be stable for a short period after its secretion since CDT was not detected from the sample from the over-grown culture (Fig. 8).

In conclusion, we produced polyclonal anti-CdtC antibody which interacts specifically with recombinant CdtC, native CdtC, or CDT holotoxin as confirmed by Western blot and ELISA. Interestingly, CDT holotoxin existed at 24h but not at 48h, meaning that CDT holotoxin was assembled at specific time during the bacterial growth.

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