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# Deletion and Purification Studies to Elucidate the Structure of the *Actinobacillus actinomycetemcomitans* Cytolethal Distending Toxin

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**Cytolethal distending toxin (CDT) is one of the exotoxins produced by *Actinobacillus actinomycetemcomitans*, an agent of localized aggressive periodontitis. We constructed N-terminal deletion mutants of CdtA using an *Escherichia coli* expression system and found that $A\Delta_{19–47}$, with a deletion from Asn-19 to Pro-47, showed comparable CDT activity but no apparent heterogeneity of CdtA. The wild-type CDT (wtCDT) and the mutant CDT ($A\Delta_{19–47}$CDT) were purified to homogeneity by introducing a histidine tag into the C-terminal end of CdtB. Both purified wtCDT and purified $A\Delta_{19–47}$CDT showed strong CDT activity and a tripartite structure composed of CdtA (subunit A), 31 kDa CdtB (subunit B), and 18.5 kDa CdtC (subunit C) in nearly a 1:1:1 stoichiometry. Importantly, subunit A was identified as heterogeneous with three CdtA variants in wtCDT, but homogeneous in $A\Delta_{19–47}$CDT. Purified CDTs also showed high stability that was absolutely dependent on the presence of sucrose in the buffer. In conclusion, the region from the Asn-19 to Pro-47 of CdtA contributes to the heterogeneous production of CdtA, but is dispensable for the toxin activity. Furthermore, this study describes an effective protocol for the purification of a native rather than reconstituted CDT, and clarifies the subunit composition of the active CDT holotoxin.**

**Key words: *Actinobacillus actinomycetemcomitans*, cytolethal distending toxin, localized aggressive periodontitis, localized juvenile periodontitis, purification.**

Abbreviations: ATM, Ataxia Telangiectasia–mutated protein; CDT, cytolethal distending toxin; LAP, localized aggressive periodontitis.

*Actinobacillus actinomycetemcomitans*, a Gram-negative small rod, is thought to be a rare member of the normal human oral commensals until individuals are edentulous. However, the amount of *A. actinomycetemcomitans* is increased in the gingival crevice of patients with localized aggressive periodontitis (LAP), which causes a rapid loss of the alveolar bone of the jaw leading to loss of the incisor or premolar. Therefore, the bacterium has long been believed to be an opportunistic pathogen for LAP (*1*). Cytolethal distending toxin (CDT) was identified recently as one of the exotoxins produced by the bacterium (*2–5*). The *A. actinomycetemcomitans* CDT has been reported to show a variety of activities. In KB (*5*), HeLa (*2*), and HEp-2 (*6*) cells, the toxin causes cell distention and cell cycle arrest at the $G_2$/M phase, and eventually elicits cell death. The toxin also blocks the proliferation of T lymphocytes to induce immunosuppression (*7*). The *A. actinomycetemcomitans* CDT likely plays an important role in the progression of LAP by promoting the destruction of periodontal tissue and inflammation of the gingiva.

CDTs are also produced by various pathogenic Gram-negative bacteria such as *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Haemophilus ducreyi*, and *Helicobacter* spp. (*8*, *9*). CDT is encoded by the gene cluster *cdtABC*. The *cdtABC* products, CdtA, CdtB, and CdtC, are secreted into the periplasm by their signal sequence, and may be assembled into the mature toxin there. Recent studies indicate that the CdtB protein contains the CDT activity (*10*), and that the other proteins, CdtA and CdtC, play roles in the entry of CdtB into the cytoplasm of a target cell (*11*, *12*). The primary structure of CdtB shows weak homology with that of DNase I, and CdtB is believed to degrade the chromosomal DNA of a target cell (*9*). Cells intoxicated with CDT exhibit phosphorylations of CDC2 and Ataxia Telangiectasia–mutated protein (ATM), and the formation of Rad50 foci and γ-H2AX (*8*, *9*, *13–15*). Subsequently, the phosphorylated ATM kinase activates RhoA, which induces the formation of actin stress fibers, resulting in the distention of the intoxicated cells (*16*). These events are thought to progress irreversibly leading to the ultimate death of the intoxicated cells.

While the action mechanism of the toxin is well elucidated, the native holotoxin structure of CDT has yet to be unambiguously determined; purification of the native CDT is known to be difficult (*8*, *9*). In this study, we report an effective purification procedure for recombinant *A. actinomycetemcomitans* CDT. Furthermore, we show that the N-terminal region of CdtA contains heterogeneous processing sites, but is dispensable to the toxin. We purified and characterized the native wild-type CDT and a mutant CDT composed of homogeneous Cdt components. While the former CDT offers evidence for the native CDT holotoxin structure, the latter can be used to determine the quaternary structure of the toxin.

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## EXPERIMENTAL PROCEDURES

*Chemicals*—Restriction endonucleases, other enzymes for DNA manipulation, and chemicals were purchased commercially.

*Bacterial Strains, Cell Lines, and Growth Media*—*E. coli* strain ER2566 (New England Biolabs, Inc., MA, USA) harboring a pTYT7 derivative was grown in Luria-Bertani (LB) broth supplemented with 50 μg/ml ampicillin. ER2566(pTYT7-cdtABC), ER2566(pTYT7), and ER2566(pTYT7-cdtBC) were constructed previously (*5*) and used as the wild-type (WT), vector control, and ΔCdtA deletion mutant in this study. pTYT7-cdtABC or its derivative plasmid is a low-copy vector derived from pBR322, and contains *cdt* genes whose expression is strictly controlled by the unique T7 promoter. The human oral epidermoid carcinoma cell line, KB, was obtained from the Health Science Research Resources Bank, Japan, and maintained in RPMI1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) containing 0.03% L-glutamine, 10% fetal bovine serum (JRH Biosciences Inc., KS, USA), and 100 μg/ml of gentamicin. For the analysis of cell distention and $G_2$/M arrest, about $5 \times 10^4$ KB cells were suspended in 1 ml of medium, placed in a well of a 12-well cell culture cluster (Corning Inc., NY, USA), and incubated for 1 d prior to the addition of CDT.

*Construction of Deletion Mutants*—An in-frame deletion mutation was introduced by a PCR method using pTYT7-cdtA-BamHI-BC (*5*) as a template. We first constructed a mutant with a deletion of Asn-19 to Ser-38 of CdtA (designated $A\Delta_{19–38}$; other deletion mutants in this study are similarly designated). The 5′-half of the DNA fragment (including the coding region for Met-1 to Ser-18 of CdtA) was amplified using 5′-CGTTCCATGGACGAACAAGCCACTAAACCC-3′ and 5′-GGCATCCTGGTCATCCAGCGG-3′, and digested with *Mlu*I and *Nco*I. The 3′-half of the *cdtA* fragment (corresponding to the coding region for Ser-39 to Asn-222 of CdtA) was amplified using 5′-GCACCCATGGCTTCAACAACACAATTCCAA-3′ and 5′-TTGGATCCTAAGCTAAGGAGTTTATATG-3′, and digested with *Nco*I and *Bam*HI. These two DNA fragments were ligated with the 6.1-kbp *Mlu*I–*Bam*HI DNA fragment from pTYT7-cdtA-BamHI-BC. The resultant plasmid was designated pTYT7-cdtA$\Delta_{19–38}$-BamHI-BC. Other deletion mutants were constructed using pTYT7-cdtA$\Delta_{19–38}$-BamHI-BC. The 3′-halves of the *cdtA* fragments were amplified with the primer 5′-TTGGATCCTAAGCTAAGGAGTTTATATG-3′ and either 5′-CAACCCATGGCCCTATTATCAAAAGCATC-3′ (for Δ19–47); 5′-TCAACCATGGTGAATTTGCTCTCTTCATCC-3′ (for Δ19–57); 5′-TCAACCATGGTGACTTTGATGGGAC-3′ (for Δ19–77); or 5′-CTGTCCATGGTCTGGGCGCTAGCAA-3′ (for Δ19–88). These DNA fragments were digested with *Nco*I and *Bam*HI. The *Nco*I-*Bam*HI DNA fragment was ligated with the 7.6-kbp *Nco*I-*Bam*HI DNA fragment from pTYT7-cdtA$\Delta_{19–38}$-BamHI-BC. The resultant plasmids were designated pTYT7-cdtA$\Delta_{19–47}$-BamHI-BC, pTYT7-cdtA$\Delta_{19–57}$-BamHI-BC, pTYT7-cdtA$\Delta_{19–77}$-BamHI-BC, and pTYT7-cdtA$\Delta_{19–88}$-BamHI-BC. After confirmation of the deletion mutations by DNA sequencing, the plasmids were introduced into *E coli* ER2566 to construct $A\Delta_{19–38}$, $A\Delta_{19–47}$, $A\Delta_{19–57}$, $A\Delta_{19–77}$, and $A\Delta_{19–88}$. An artificial sequence was inserted at the deletion point (Met-Ala in $A\Delta_{19–38}$ and $A\Delta_{19–47}$, Met-Val in $A\Delta_{19–57}$ and $A\Delta_{19–77}$, and Met in $A\Delta_{19–88}$) owing to the insertion of an *Nco*I recognition sequence CCATGG for the construction of deletion mutants.

*Construction of Plasmids for the Purification of CDT*—The DNA fragment containing *cdtA* and *cdtB* was amplified by PCR using the primers 5′-AAGGATCCGTGATGGTGATGGTGATGGCGATCATGAACAAAACT-3′ and 5′-GGCATCCTGGTCATCCAGCGG-3′, and pTYT7-cdtAB-BamHI-C (*5*) as a template. The amplified DNA fragment was digested with *Mlu*I and *Bam*HI, and ligated with the 6.6-kbp *Mlu*I–*Bam*HI DNA fragment from pTYT7-cdtAB-BamHI-C. The resultant plasmid, designated pTYT7-cdtAB-His-BamHI-C, contains a gene for a histidine tag inserted into the 3′-terminal end of the *cdtB* of pTYT7-cdtAB-BamHI-C. Then, the 1.6-kbp *Mlu*I–*Nhe*I DNA fragment from pTYT7-cdtA$\Delta_{19–47}$-BamHI-BC was ligated to the 6.3-kbp *Mlu*I–*Nhe*I DNA fragment from pTYT7-cdtAB-His-BamHI-C. The resultant plasmid was designated pTYT7-cdtA$\Delta_{19–47}$-B-His-BamHI-C. CdtB-His contains an additional sequence, $(His)_6$-Gly-Ser, at the C-terminal end of CdtB. The plasmid was introduced into ER2566 to construct ER2566(pTYT7-cdtAB-His-BamHI-C) or ER2566(pTYT7-cdtA$\Delta_{19–47}$-B-His-BamHI-C).

*Preparation of Crude Extract*—Cells overexpressing CDT were harvested, suspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.4 mM KH2PO4) supplemented with 0.2 mM of phenylmethanesulfonyl fluoride, sonicated, and centrifuged (10,000 × *g,* 30 min). The supernatant was filtered (0.45 μm) and used immediately as a "crude extract".

*Purification of Recombinant CDT*—Cells overexpressing CDT were harvested, washed with PBS, suspended in buffer [30 mM Tris-HCl pH 8.0, 20% (w/v) sucrose, 4 mM EDTA, 1 mg/ml of lysozyme, and 0.2 mM phenylmethanesulfonyl fluoride], incubated for 15 min on ice, and centrifuged (17,600 × *g,* 15 min). The supernatant was filtered (0.45 μm). EDTA, lysozyme, and phenylmethanesulfonyl fluoride were removed from the filtrate by three rounds of concentration-dilution on an ultrafiltration membrane XM50 (Millipore Co. Bedford, MA, USA). The final concentrated sample was diluted with wash buffer (30 mM Tris-HCl pH 8.0, 10% [w/v] sucrose, and 200 mM NaCl) and applied to a column filled with $Ni^{2+}$-chelating Sepharose Fast Flow (Amersham Pharmacia Biotech AB, Sweden) equilibrated with the wash buffer. The column was washed with wash buffer containing 50 mM imidazole and then 100 mM imidazole. Purified CDT was eluted with wash buffer containing 200 mM imidazole. Imidazole and sodium chloride were removed from the eluate by concentration-dilution (three rounds) on an ultrafiltration membrane YM50 (Millipore Co. Bedford, MA, USA). The purified samples were stored at –20°C for later use.

*Determination of Subunit Stoichiometry*—The densities of protein bands in 15% SDS-PAGE gels stained with Coomassie Brilliant Blue R-250 were determined using a Luminescent Image Analyzer LAS-1000plus (Fuji Photo Film Co., Ltd., Japan) and Image Gauge ver. 3.1.2 (Fuji Photo Film Co., Ltd., Japan). Data were collected from more than 5 gels and averaged. The molecular weight of each subunit was determined by 15% SDS-PAGE analysis.


Fig. 1. **Alignment of the N-terminal halves of CdtA proteins.** The primary structure of the *A. actinomycetemcomitans* CdtA (Met-1 to Gly-120) was compared with those of other CdtA proteins. Conserved amino acids are boxed. The putative Lipobox is shaded and the cysteine residue for lipid modification is marked by #. Deletions in the CdtA mutants are indicated above the alignment. A non-homologous region, from Thr-75 to Val-111 (TSNSANNNAANGINPRFKDEAFNDVLIFENRPAV), found in the *C. jejuni* CdtA, is not described, but the position of the sequence is indicated by [+]. A.act., CdtA (Met-1 to Gly-120) of *A. actinomycetemcomitans* ATCC29522 (DDBJ, AB017807); H.duc., CdtA (Met-1 to Gly-120) of *H. ducreyi* 35000 (GenBank, U53215), C.jej., CdtA (Met-1 to Leu-157) of *C. jejuni* 81–176 (GenBank, U03293); E.co-1, CdtA (Met-1 to Gly-106) of *E. coli* 6468/62 (O86:H34) (GenBank, U04208); E.co-2, CdtA (Met-1 to -123) of *E. coli* 9142-88 (O128:H$^-$) (GenBank, U04208).

*Determination of the N-Terminal Amino Acid Sequence*—Cdt subunits of the purified CDT (10 μg of protein) were separated by 15% SDS-PAGE and transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad Lab., CA, USA). Proteins were visualized by staining with Coomassie Brilliant Blue R-250 and excised. The N-terminal amino acid sequence of the stained protein band was determined with a PPSQ-10 gas phase peptide sequencer system (Shimadzu Co., Japan).

*Determination of CDT Activity*—KB cells were treated with CDT, incubated for 2 d, and used for the analysis of the CDT activity. For the analysis of cell distention, KB cells were directly observed using a DIAPHOTO-TMD phase contrast inverted microscope (Nikon Co., Japan) at ×200 magnification. For the analysis of $G_2$/M arrest, KB cells were collected, fixed in 1% formaldehyde in PBS, washed with PBS, labeled with propidium iodide using the CycleTest PLUS DNA Reagent Kit (Becton Dickinson and Co., CA, USA), and examined using FACScan (Becton Dickinson and Co., CA, USA). The $G_2$/M content of the cells was analyzed using ModFit version II (Becton Dickinson and Co., CA, USA). The CDT activity was determined from the results of at least three independent experiments.

*Rapid Molecular-Sieve Chromotography*—Purified CDT (100 μg) was subjected to gel filtration HPLC analysis using TSK-GEL G3000SW$_{XL}$ (Tosoh Co., Tokyo, Japan) with 30 mM Tris-HCl pH 7.4, 10% (w/v) sucrose, and 250 mM NaCl as the running buffer. The eluted proteins were detected by SPD-6A and recorded on a C-R6A (Shimadzu Co., Japan). Gel filtration standards (Bio-Rad Lab., CA, USA) were prepared according to the manual and used as the standard (200 μg of 670 kDa thyroglobulin,, 200 μg of 158 kDa bovine gamma globulin, 200 μg of 44 kDa chicken ovalbumin, 100 μg of 17 kDa equine myoglobin, and 20 μg of vitamin B-12).

*Other Methods*—PCR was performed with a Programmable Thermal Controller PTC-100 (MJ Research, Inc., MA, USA) using Vent DNA polymerase (New England Biolabs, Inc., MA, USA). Protein concentration was determined with a BCA Protein Assay Kit (Pierce, IL, USA) using bovine serum albumin as the standard. In western blot analysis, proteins were separated by 15% SDS-PAGE, transferred to a 0.45 μm nitrocellulose membrane (Millipore Co. Bedford, MA, USA) with a semi-dry type transfer apparatus (AE-6670, ATTO Co. Japan), and visualized using anti-rabbit IgG goat IgG-conjugated HRP (Bio-Rad Lab., CA, USA) as a secondary antibody.

## RESULTS

*Construction and Characterization of CdtA N-Terminal Deletion Mutants*—We previously reported that CdtA is processed between Pro-47 and Leu-48 of the purified reconstituted-CDT (*5*). Similar processing of CdtA may occur also in the CDT-producing strain, and so we constructed a mutant with an in-frame deletion in the N-terminal region of CdtA. We constructed five CdtA deletion mutants, designated A$\Delta_{19–38}$, A$\Delta_{19–47}$, A$\Delta_{19–57}$, A$\Delta_{19–77}$, and A$\Delta_{19–88}$ (Fig. 1). The results of each deletion were investigated using crude extracts prepared from the mutants. As summarized in Table 1, cell distending activity was not affected in A$\Delta_{19–47}$ and was slightly increased in A$\Delta_{19–38}$ compared with the wild-type (WT), ER2566(pTYT7-cdtABC). In contrast, the activity was reduced to about 10% and 1% in A$\Delta_{19–57}$ and A$\Delta_{19–77}$, respectively, compared with WT. In A$\Delta_{19–88}$, the activity was less than 0.1% of that of the WT and was undetectable. These results indicate that the N-terminal region from the Asn-19 to Pro-47 of CdtA is not required for the cell distending activity. Next, the expression of each Cdt component was investigated by western blot analysis using anti-CdtA, anti-

Table 1. **Summary of the cell distending activity of CdtA deletion mutants.**

| | Added crude extract (μg) | | | | | | Relative activity (%) |
|---|---|---|---|---|---|---|---|
| | 100 | 10 | 1 | 0.1 | 0.01 | 0.001 | |
| WT | + | + | + | + | +/– | – | 100 |
| Vector | – | – | – | – | – | – | <0.1 |
| $A\Delta_{19–88}$ | – | – | – | – | – | – | <0.1 |
| $A\Delta_{19–77}$ | + | + | – | – | – | – | 1 |
| $A\Delta_{19–57}$ | + | + | + | – | – | – | 10 |
| $A\Delta_{19–47}$ | + | + | + | + | +/– | – | 100 |
| $A\Delta_{19–38}$ | + | + | + | + | + | – | >100 |
| WT-His[a] | + | + | + | + | +/– | – | 100 |
| $A\Delta_{19–47}$His[b] | + | + | + | + | +/– | – | 100 |

KB cells grown in 1 ml of medium were treated with the indicated amount of crude cell extract and incubated for 2 d. +, cell distending positive; –, cell distending negative; +/–, partial cell distending activity. [a]ER2566(pTYT7-cdtAB-His-BamHI-C). [b]ER2566(pTYT7-cdtA$\Delta_{19–47}$-B-His-BamHI-C).

CdtB, or anti-CdtC antiserum. As shown in Fig. 2a, heterogeneous CdtA bands were detected in the WT (as 24.5 kDa, 19.5 kDa, and 18 kDa bands; lane 3) and in $A\Delta_{19–38}$ (as 21 kDa, 19.5 kDa, and 18 kDa bands; lane 4), whereas an apparent homogeneous CdtA band was detected in $A\Delta_{19–47}$ (an 18.5 kDa band with a minor 21 kDa band; lane 5) and AΔ19–77 (a 17.5 kDa band; lane 7). The CdtA expression levels were severely reduced in both $A\Delta_{19–57}$ and $A\Delta_{19–88}$ (Fig. 2a, lanes 6 and 8). On the other hand, in all crude extracts, CdtB was detected as a homogeneous 30 kDa band with a minor 31.5 kDa band and/or a minor 29 kDa band (Fig. 2b), and CdtC was detected as a homogeneous 18.5 kDa band (Fig. 2c). However, the expression levels of CdtB and CdtC were reduced in $A\Delta_{19–88}$ and the ΔCdtA deletion mutant (lanes 8 and 2 of Fig. 2b and c). These results of western blot analysis indicate that the deletion of the N-terminal region from the Asn-19 to Pro-47 of CdtA specifically diminishes the heterogeneity of CdtA but does not affect the expression of the Cdt components. Therefore, the region covering Asn-19 to Pro-47 of CdtA is dispensable for the biogenesis of CDT.

*Purification and Characterization of CDTs*—For the purification of CDT, we introduced a histidine tag into the C-terminal end of CdtB. ER2566(pTYT7-cdtAB-His-BamHI-C), which produces CdtA, CdtB fused with a histidine tag (CdtB-His), and CdtC, was constructed for the purification of the wild-type CDT (wtCDT). ER2566(pTYT7-cdtA$\Delta_{19–47}$-B-His-BamHI-C), a derivative of ER2566(pTYT7-cdtAB-His-BamHI-C), was constructed for the purification of the mutant CDT ($A\Delta_{19–47}$CDT). These strains show comparable cell distending activity to their original strains (Table 1), indicating that the introduction of a histidine tag does not affect the CDT activity. The CDTs produced in these strains were purified to homogeneity by the metal-affinity purification protocol. The purified samples, wtCDT and $A\Delta_{19–47}$CDT, were analyzed by 15% SDS-PAGE. wtCDT contains several protein bands, *i.e.*, 31 kDa, 24.5 kDa, 18.5 kDa, and 18 kDa bands (Fig. 3A), whereas $A\Delta_{19–47}$CDT shows two protein bands, 31 kDa and 18.5 kDa bands (Fig. 4A). These protein bands were identified by western blot analysis using anti-CdtA antiserum, anti-CdtB antiserum, or anti-CdtC antiserum. In wtCDT, CdtA was detected as heterogeneous bands (24.5 kDa and 18 kDa bands shown in Fig. 3B-a), while CdtB, and CdtC were detected as 31 kDa (Fig. 3B-b) and 18.5 kDa bands (Fig. 3B-c), respectively. Therefore, the 24.5 kDa and 18 kDa bands detected in the SDS-PAGE gel are CdtA, and designated as subunit A of wtCDT (Fig. 3A), and the 31 kDa protein and the 18.5 kDa protein are CdtB and CdtC, and are designated as subunit B and subunit C of wtCDT (Fig. 3A). By contrast, CdtA, CdtB, and CdtC of $A\Delta_{19–47}$CDT were detected as 18.5 kDa, 31 kDa and 18.5 kDa bands (Fig. 4B-a–c). Similarly, the 18.5 kDa CdtA, 31 kDa CdtB and 18.5 kDa CdtC were designated as subunit A, subunit B, and subunit C of $A\Delta_{19–47}$CDT. Using our protocol, about 0.06 and 0.2 mg of purified CDT protein was obtained from a 1 liter culture of ER2566(pTYT7-cdtAB-His-BamHI-C) and ER2566(pTYT7-cdtA$\Delta_{19–47}$-BamHI-BC), respectively, with

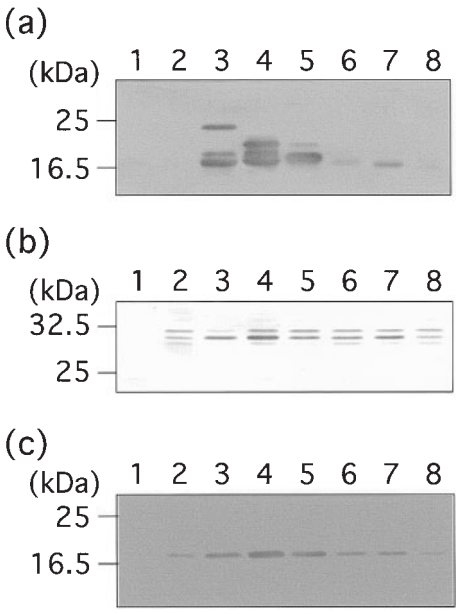


Fig. 2. **Western blot analysis of the cell extract of the CdtA deletion mutant.** The crude cell extract (10 μg) was separated by 15% SDS-PAGE followed by western blot analysis using anti-CdtA antiserum (a), anti-CdtB antiserum (b) or anti-CdtC antiserum (c). Lane 1, vector control; lane 2, ΔCdtA; lane 3, WT; lane 4, $A\Delta_{19–38}$; lane 5, $A\Delta_{19–47}$; lane 6, $A\Delta_{19–57}$; lane 7, $A\Delta_{19–77}$; lane 8, $A\Delta_{19–88}$.

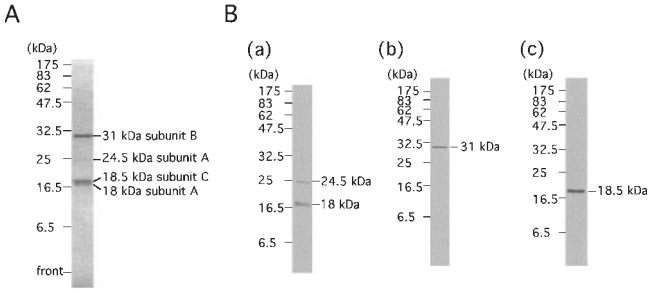


Fig. 3. **SDS-PAGE and western blot analyses of wtCDT.** wtCDT (2 μg) was separated by 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250 (A) or western blot analysis (B) using anti-CdtA antiserum (a), anti-CdtB antiserum (b) or anti-CdtC antiserum (c).

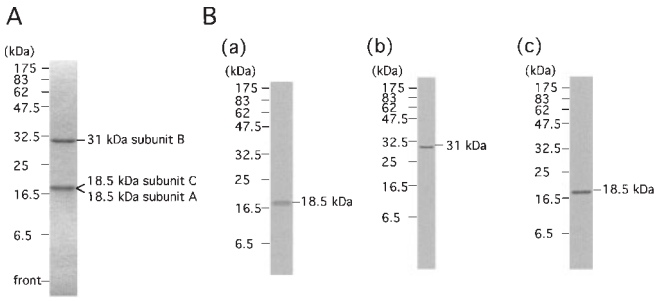


Fig. 4. **SDS-PAGE and western blot analyses of A$\Delta_{19-47}$CDT.** A$\Delta_{19-47}$CDT (2 μg) was separated by 15% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250 (A) or western blot analysis (B) using anti-CdtA antiserum (a), anti-CdtB antiserum (b) or anti-CdtC antiserum (c).

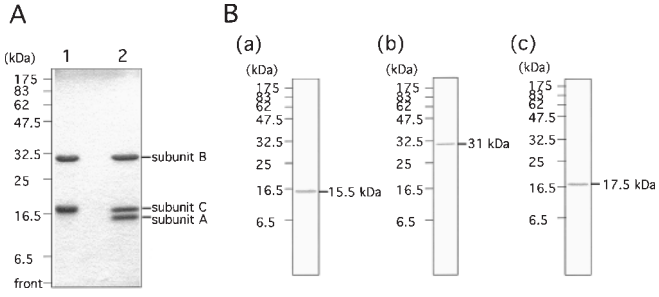


Fig. 5. **Non-reduced SDS-PAGE and western blot analyses of A$\Delta_{19-47}$CDT.** A$\Delta_{19-47}$CDT dissolved in sample buffer (65 mM Tris-HCl pH 6.8, 3% SDS, and 10% glycerol) (A lane 2 and B) or sample buffer supplemented with 0.1 M DTT (A lane 1) was incubated at 95°C for 5 min. A: A 5 μg of the sample was separated by 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. B: A 0.5 μg of the sample was separated by 15% SDS-PAGE and subjected to western blot analysis using anti-CdtA antiserum (a), anti-CdtB antiserum (b) and anti-CdtC antiserum (c).

more than a third (33 to 69%) of the total cell distending activity of the original periplasmic fraction recovered.

*Assignment of Cdt Subunits*—The Cdt subunits were further assigned based on Edman degradation analysis. The results are summarized in Table 2. The 31 kDa subunit B, derived from wtCDT or A$\Delta_{19-47}$CDT, shows the sequence "NLSDFKVA," which is identical to the sequence from Asn-23 to Ala-30 of CdtB. The theoretical molecular mass (MM) of the CdtB-His protein from Asn-23 to the last residue of CdtB-His is calculated to be about 29.8 kDa, nearly 31 kDa. Similarly, the 18.5 kDa subunit C of the purified CDTs shows the sequence "ESNPDPTT", which is identical to the sequence from Glu-21 to Thr-28 of CdtC. The theoretical MM of Glu-21 to the last residue, Ser-186, of the CdtC protein is calculated to be about 18.4 kDa, nearly 18.5 kDa. Therefore, subunit B or subunit C of the purified CDTs is homogeneous with only one form of CdtB or CdtC. In contrast to subunit B and subunit C, subunit A was identified as heterogeneous with CdtA variants in wtCDT. In wtCDT, the 18 kDa subunit A shows two sequences "LLSSSKNG" and "LSSSKNGQ." These sequences are identical to the Leu-59 to Gly-66 and Leu-60 to Gln-67 of CdtA. The theoretical MM of the CdtA proteins from Leu-59 to the last residue (Asn-222) and Leu-60 to Asn-222, are calculated to be about 18.2 kDa and 18.1 kDa, nearly 18 kDa. The N-terminal sequence of the 24.5 kDa subunit A was not determined since it showed no amino acid signal. Therefore, subunit A of wtCDT contains at least three CdtA variants. A$\Delta_{19-47}$CDT shows only one form of subunit A, the 18.5 kDa CdtA protein, which has only one sequence, "SSMPLNLL." The sequence is identical to the sequence spanning Ser-53 to Leu-60 of CdtA. The theoretical MM of Ser-53 to Asn-222 of the CdtA protein is calculated to be about 18.8 kDa, nearly 18.5 kDa. Therefore, subunit A of A$\Delta_{19-47}$CDT is homogeneous with only one form of CdtA.

*Molar Stoichiometry of Cdt Subunits of Purified CDTs*—The molar stoichiometry of the Cdt subunits was determined from the density of protein bands in the SDS-PAGE gel stained with Coomassie Brilliant Blue R-250. In wtCDT, the molar stoichiometry for the 24.5 kDa subunit A, 18 kDa subunit A, subunit B, and subunit C (Fig. 3A) was determined to be 0.19:0.84:1:1.06. Therefore, the native wild-type CDT complex is composed of subunit A, subunit B, and subunit C in a 1.03:1:1.06 stoichiometry, nearly 1:1:1. In A$\Delta_{19-47}$CDT, the molar stoichiometry of the 18.5 kDa protein (containing subunit A and subunit C) and subunit B (Fig. 4A) was determined to be 2.04:1. However, in non-reduced SDS-PAGE analysis, A$\Delta_{19-47}$CDT showed three protein bands, 31 kDa, 17.5 kDa, and 15.5 kDa bands (Fig. 5A, lane 2), which were identified as CdtB, CdtC, and CdtA by western blot analysis (Fig. 5B, a–c). The molar stoichiometry for the 15.5 kDa subunit A, 31 kDa subunit B, and 17.5 kDa subunit C in a non-reduced SDS-PAGE gel was determined to be 1.02:1:1.01, nearly 1:1:1.

*The CDT Activity of Purified CDTs*—We determined the cell distending activity and the $G_2$/M arrest activity of the purified CDTs. As shown in Fig. 6, 0.75 ng of purified CDT protein was enough to evoke cell distention and cell cycle arrest at $G_2$/M, indicating that wtCDT and A$\Delta_{19-47}$

Table 2. **Summary of the peptide sequences of CDT subunits.**

| Purified CDT | Subunit | MM (kDa) | Identified sequence | Corresponding sequence | Theoretical MM (kDa) |
|---|---|---|---|---|---|
| wtCDT | A | 24.5 | nd[a] | — | 22.9[b] |
| | | 18 | LLSSSKNG | 59–66 of CdtA | 18.2 |
| | | | LSSSKNGQ | 60–67 of CdtA | 18.1 |
| | B | 31 | NLSDFKVA | 23–30 of CdtB | 29.8 |
| | C | 18.5 | ESNPDPTT | 21–28 of CdtC | 18.4 |
| A$\Delta_{19-47}$CDT | A | 18.5 | SSMPLNLL | 53–60 of CdtA | 18.8 |
| | B | 31 | NLSDFKVA | 23–30 of CdtB | 29.8 |
| | C | 18.5 | ESNPDPTT | 21–28 of CdtC | 18.4 |

[a]Not detected. [b]MM of the full-length product.

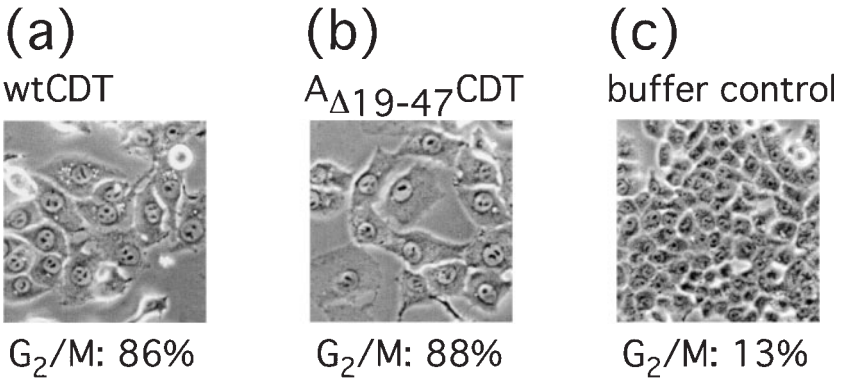


Fig. 6. **The CDT activity of purified CDTs.** KB cells were treated with buffer (c), 0.75 ng of wtCDT (a), or 0.75 ng of $A\Delta_{19-47}$CDT (b), and incubated for 2 d. Distending cells are shown. The $G_2$/M content of the intoxicated KB cells is shown below the photo.

CDT have comparable CDT activities. These intoxicated KB cells disintegrated within a week (data not shown).

*Effect of Sucrose on the Stability of Purified CDTs*— The CDT activity of the purified CDT samples dissolved in dilution buffer (30 mM Tris-HCl pH 8.0 and 10% sucrose) was unaffected after several rounds of freeze-thawing, storage at –20°C for several months, or storage at 4°C for at least 2 mo (data not shown). By contrast, the activity of the samples dissolved in the buffer but 0.1% sucrose was remarkably reduced after one round of freeze-thawing or storage at 4°C for 3 or 7 d (Fig. 7).

## DISCUSSION

CdtA is known to be heterogeneously produced in the crude cell extract of *A. actinomycetemcomitans* or recombinant *E. coli* (*2*), suggesting that additional processing of CdtA occurs in the normal biogenesis of CDT. In the present study, deletion of the region from Asn-19 to Pro-47 of CdtA results in the homogeneous production of CdtA without loss of CDT activity. The N-terminal region spanning Asn-19 to Asn-75 of the *A. actinomycetemcomitans* CdtA is very poorly conserved among CdtA proteins (Fig. 1). Interestingly, the dispensable region from Asn-19 to Pro-47, and all of the processing sites of subunit A (Table 2), are located within the non-conserved region. Identification of proteases involved in additional processing of the *A. actinomycetemcomitans* CdtA may clarify the mechanism of the maturation of CdtA. Downstream of the non-conserved region, we found a conserved region (Met-80 to Gly-120 of the *A. actinomycetemcomitans* CdtA, as shown in Fig. 1). Since $A\Delta_{19-77}$ and $A\Delta_{19-88}$ showed little cell distending activity (Table 1), the conserved region is likely to be important for *A. actinomycetemcomitans* CdtA. Deletion of the region of the *C. jejuni* CdtA results in defects in holotoxin assembly of the *C. jejuni* CDT (*11*).

There are several reports describing attempts to purify CDT using conventional chromatographic methods (*7*, *17*), molecular-sieve chromatography followed by immunoaffinity-purification (*18*, *19*), or metal-affinity chromatography of histidine-tagged CDT (*18*, *20*), but the resultant preparations apparently showed poor purity, weak activity, and/or decomposition of the holotoxin structure.

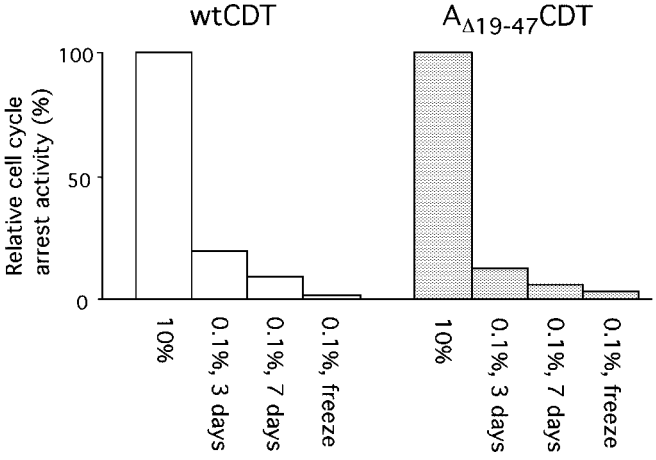


Fig. 7. **Effect of sucrose on stabilizing the activity of purified CDTs.** wtCDT, and $A\Delta_{19-47}$CDT (25 μg/ml) dissolved in 30 mM Tris-HCl pH 8.0 containing 10 or 0.1% sucrose ("10%"and "0.1%," respectively) were incubated at 4°C for 3 d ("3 days"), at 4°C for 7 d ("7 days") or at –20°C for 7 d ("freeze"), and used for the analysis of $G_2$/M arrest of KB cells. The $G_2$/M content of KB cells treated with buffer (about 13%) was subtracted from the content of KB cells intoxicated with 0.25 ng of the incubated CDT sample, and the resultant content was defined as "relative cell cycle arrest activity." Note that the $G_2$/M content of KB cells treated with 0.25 ng of CDT sample ("10% sucrose") was about 51% and was unaffected by the incubation.

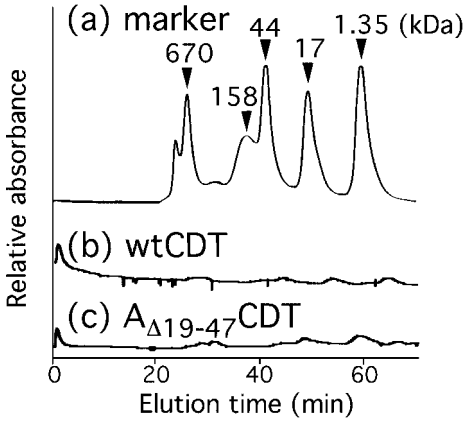


Fig. 8. **Gel filtration profiles of purified CDTs.** Standard markers (a), 100 μg of wtCDT (b), and 100 μg of $A\Delta_{19-47}$CDT (c) were applied to a gel filtration HPLC column TSK-GEL $G3000SW_{XL}$. Eluted proteins were detected by monitoring absorbance at 280 nm. Molecular mass of standards is shown in (a). In (b) and (c), the relative absorbance is shown magnified 10-fold.

We believe that the failure to purify CDT was mainly due to the very low stability of the toxin. In the present study, CdtA, CdtB-His, and CdtC were overproduced in a recombinant *E. coli* strain, and the histidine-tagged CDT holotoxin was purified to homogeneity using $Ni^{2+}$-chelating Sepharose with buffers supplemented with sucrose. It should be stressed that sucrose greatly stabilizes CDT (Fig. 7), indicating that sucrose is required for the purification of CDT. The purified wtCDT shows strong CDT activity. The specific activity for the distention of KB cells was about 100 times higher than that of the crude cell extract (Table 1 and Fig. 6).

CDT is believed to be a tripartite toxin composed of CdtA, CdtB, and CdtC in a 1:1:1 stoichiometry (*9*), according to the results of the subunit stoichiometry of the purified reconstituted-CDTs (*5*, *21*); however, no report has yet provided conclusive evidence about the subunit composition of a native CDT. Here, we show that wtCDT is composed of heterogeneous forms of subunit A (24.5 kDa CdtA and 18 kDa CdtA), subunit B (31 kDa CdtB), and subunit C (18.5 kDa CdtC) in a nearly 1:1:1 stoichiometry. We were unable to determine the molecular mass of the purified CDT complex because the purified CDT sample likely adsorbed to the rapid molecular-sieve chromatography HPLC column, even when the running buffer contained sucrose (Fig. 8b and c). Purification of CDTs is complicated by the fact that the toxin appears to aggregate (*8*), and the presence of sucrose in the buffer is not sufficient for the purification of CDT by a standard chromatographic approach as reported (*5*). However, we believe that a homogeneous CDT holotoxin was obtained by our purification protocol since contamination by CdtB-His (*e.g.*, a homo-multimer form of CdtB-His) in the purified CDT sample can be excluded because CdtB-His passed through or adsorbed to the ultrafiltration membrane in the absence of CdtA and CdtC (data not shown). In wtCDT, three variants of subunit A were identified (Table 2). The results of N-terminal amino acid analyses of the Cdt subunits were identical with those reported by Shenker *et al.* recently (*20*). Importantly, subunit A of the purified wild-type *A. actinomycetemcomitans* CDT is heterogeneous. We also purified the CDT from ER2566(pTYT7-cdtA$\Delta_{19-38}$-B-His-BamHI-C) (Table 1). The purified A$\Delta_{19-38}$CDT shows properties similar to those of wtCDT and A$\Delta_{19-47}$CDT, but the identified CdtA proteins were heterogeneously processed to a greater extent than in the case of wtCDT (data not shown).

In contrast, A$\Delta_{19-47}$CDT is composed of homogeneous Cdt components: an 18.5 kDa subunit A, 31 kDa subunit B, and 18.5 kDa subunit C (Table 2) in a nearly 1:1:1 stoichiometry (Fig. 5A, lane 2), while it shows CDT activity similar to that of wtCDT. This indicates that the deletion mutation in A$\Delta_{19-47}$CDT eliminates the heterogeneity of subunit A without loss of CDT activity. While four cysteine residues are present in mature CdtA and mature CdtC (CdtB contains no cysteine residues), the results of non-reduced SDS-PAGE analysis suggests that no inter-molecular disulfide bonds are present in the Cdt subunits of A$\Delta_{19-47}$CDT (Fig. 5B). The purified A$\Delta_{19-47}$CDT sample can be concentrated to about 50 mg/ml without loss of CDT activity. The properties of A$\Delta_{19-47}$CDT likely satisfy those necessary for crystallization

In conclusion, we report the purification and characterization of the native *A. actinomycetemcomitans* CDT holotoxin. Our purification protocol may be applicable to other CDTs. Furthermore, we succeeded in purifying A$\Delta_{19-47}$CDT, which is composed of homogeneous Cdt components. We are now interested in determining the crystal structure of A$\Delta_{19-47}$CDT.

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