Biogenesis of *Actinobacillus actinomycetemcomitans* Cdt holotoxin

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Summary

The cell cycle G2/M specific inhibitor, Cytolethal Distending Toxin (Cdt), from *Actinobacillus actinomycetemcomitans* is composed of CdtA, CdtB, and CdtC coded on the *CdtA*, *CdtB* and *CdtC* genes that are tandem on the chromosomal *cdt* locus. *A. actinomycetemcomitans* CdtA has the lipid binding consensus domain, the so-called "lipobox", at the N-terminal signal sequence. Using *E.coli* carrying pTK3022, we show the sixteenth residue, cysteine, of CdtA bound [^{3}H]palmitate or [^{3}H]glycerol. Further post translational processing of the signal peptide, CdtA, was inhibited using globomycin, an inhibitor of lipoprotein-specific signal peptidase II. Fractionation and immunoblot show the lipid modified CdtA is present in the outer membrane. Immuno-precipitation and the pull-down assay of the Cdt complex from *E. coli* carrying a plasmid containing *CdtABC* demonstrated the Cdt complex in the periplasm is composed of CdtA, CdtB, and CdtC, and that the Cdt complex in culture supernatant is a N-terminally truncated (36-43 amino acids) form of CdtA (CdtA'), CdtB, and CdtC. This suggests Cdt is present as a complex both in the periplasm and the supernatant where CdtA undergoes post-translation processing to CdtA' in the process of biogenesis and secretion of Cdt holotoxin into the culture supernatant. Site-directed mutagenesis of the sixteenth cysteine residue to glycine in CdtA altered localization of CdtA in the cell and reduced the amount of Cdt activity in the culture supernatant. This suggests Cdt forms a complex inside the periplasm for lipid modification where post-translational processing of CdtA plays an important role for the efficient production of Cdt holotoxin into the culture supernatant.

Introduction

Cytolethal distending toxin (Cdt) is a cell cycle G2/M specific inhibitor produced by several pathogenic bacteria including *Campylobacter* spp. (16, 39), *Escherichia coli* (17, 28, 29, 33), *Shigella dysenteriae* (26), *Haemophilus ducreyi* (6, 10), *Helicobacter hepaticus* (4, 41), *Salmonella typhi* (13), and *Actinobacillus actinomycetemcomitans* (20, 37, 38). Cdt-poisoned eukaryotic cells show cell cycle arrest and subsequent cellular distension followed by cell death (16, 17, 24, 28, 30). Except for *Salmonella typhi*, Cdt is coded on the *CdtA*, *CdtB*, and *CdtC* genes that are tandemly-located at the chromosomal *cdt* locus; and the expression of the three components CdtA, CdtB, and CdtC are necessary for full toxicity (2, 19, 31, 36). Cdt is suggested to be a unique tripartite AB toxin in which CdtB is the active'A' subunit and CdtA and CdtC constitute the heterodimeric 'B' subunit. Recently, the crystal structure of *H. ducreyi* Cdt confirms this structure (21). The structure Cdt holotoxin consists of CdtA, CdtB, and CdtC forming a ternary complex in a 1:1:1 stoichiometry. CdtA and CdtC form ricin-like lectin domains that may play a role as recognizing the cellular receptor that delivers the CdtB subunit into the target cells. CdtB shares conserved residues with the active sites of phosphodiesterase namely DNase-I and sphingomyelinase and is structurally similar to the DNase-I fold. Despite the absence of direct evidence that CdtB acts as DNase on chromosomal DNA *in vivo*, accumulating circumstantial evidence indicates CdtB induces DNA damage inside the target nucleus (11, 18). DNA damage by CdtB may activate the checkpoint control, PI3 kinase protein ATM (Ataxia Telangiectasia Mutated) that is responsible for activating the phosphorylation of the downstream checkpoint protein kinase Chk2 (1, 8). Activated Chk2 phospholylates the phosphatase, Cdc25, promotes its binding to the 14-3-3 protein and subsequent sequestration in the cytoplasm (5, 9). Cdc25C is then unable to

dephosphorylate and activate the nuclear complex Cdk1-cyclin B which is the universal mitosis inducer in eukaryotes (7, 12, 34). Consequently, Cdk1 is maintained in the inactive tyrosine-phosphorylated state and the cells exposed to Cdt remain arrested in the G2 phase of the cell cycle (30).

In spite of the accumulation of knowledge on Cdt effect on target cells, less information is available on biogenesis of Cdt holotoxin. *A. actinomycetemcomitans* CdtA, CdtB, and CdtC are translated as approximately 25, 32, and 21 kDa proteins, respectively, and are presumably exported across the cytoplasmic membrane with the cleavage of signal sequences using signal peptidases. We previously show *A. actinomycetemcomitans* CdtA possesses a putative lipid modification motif (38); but have not characterized CdtA for the lipid modification. Since genetic manipulation of *A. actinomycetemcomitans* is difficult, we characterized CdtA using *E.coli* carrying the *A. actinomycetemcomitans cdtABC* genes to extrapolate the biogenesis of Cdt holotoxin in *A. actinomycetemcomitans*. Here we show membrane-associated CdtA is a lipo-protein. In the periplasm, Cdt is a complex composed of CdtA, CdtB, and CdtC. Whereas, Cdt in the culture supernatant is a complex composed of N-terminally truncated CdtA (CdtA'), CdtB, and CdtC. This suggests CdtA undergoes lipid-modification during the export process and subsequent N-terminal processing after forming a complex with CdtB and CdtC in the periplasm. We suggest lipid modification of CdtA is important for the export of *A. actinomycetemcomitans* Cdt holotoxin into the culture supernatant.

Materials and Methods

Bacterial strains and growth media. Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* was grown aerobically in Luria-Bertani (LB) medium or on LB agar plates. *A. actinomycetemcomitans* was grown in Trypticase Soy Broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) supplemented with 1% (wt/vol) yeast extract in a 5% CO_2 atmosphere. Ampicillin (50 µg/ml) or kanamycin (50 µg/ml) was added when necessary.

HeLa cell culture. HeLa cells (ATCC CCL2) were cultured in Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum at 37˚C in a 5%CO_2/95% air atmosphere.

Assay for Cdt activity. The test samples were prepared from the culture supernatant and the cell lysate of *E. coli* carrying pMW*cdtABC* or pMW*cdtA*^{C16G}*BC*. Exponentially growing cells at OD_{660} = ca. 0.5 were harvested using centrifugation at 5,000 x *g* for 10 min and the culture supernatant was recovered. Cell lysates were prepared from the harvested cells using the method described below. The protein concentration of culture supernatant and cell lysate were measured using the BioRad protein assay kit and adjusted respectively by adding LB to culture supernatant or by adding PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 at pH7.3) to the cell lysate. HeLa cells were plated on a 96-well plate (Falcon; Becton Dickenson) at a concentration of 2 x 10^3 cells per 100 µl well one-day before the experiment. One hundred microliters of filter-sterilized (0.22 µm pore size filter) sample containing 0.5 µg of total protein from the culture supernatant or 100 µg of total protein from the cell lysate were inoculated into the HeLa cell monolayer in the first well of the 96-well plate. After mixing well, a half aliquot (100 µl) of sample-medium mixture was added to the next well and 1:2 serially diluted likewise through 12 dilutions. Morphological change was observed by phase contrast microscopy (Nikon DIAPHOT

300) from day 1 to day 3. Cell distension was defined as more than 5 times the expansion in size as compared to that of control cells. Cyto-distension activity (total activity) was titrated using the end point as the highest two-fold dilution of toxic material giving 50% transformed cells after 72 h incubation (CD_{50}). Specific activity was defined as the CD_{50}/mg of the total protein (CD_{50}/mg).

Preparation of crude Cdt. Cdt holotoxin was obtained from *A. actinomycetemcomitans* Y4 or *E. coli* carrying the *cdtABC* genes on the plasmids, pTK3022 or pQE*cdtABC* (23, 38). Cdt expression in *E. coli* was induced by the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 4 h at OD_{660}= 0.5 - 0.7. Crude Cdt from *A. actinomycetemcomitans* or the recombinant *E. coli* was prepared in several fractions: 1) culture supernatant: Cells exponentially growing in culture medium was inoculated into 3 l of fresh medium and incubated with continuous agitation using a rotary shaker for 4 h at 37˚C until the cells reached the stationary phase. The culture was centrifuged at 5,000 x *g* for 30 min at 4˚C. The concentrated culture filtrate was prepared using 80% saturated ammonium sulfate precipitation of the culture supernatant followed by dialysis with PBS. The dialyzed sample was filter sterilized through a membrane filter (pore size, 0.22 μm; Millipore). 2) periplasm: Cells recovered from culture by centrifugation were washed twice with PBS and pelleted by centrifugation. The pellet was suspended in 0.2 M Tris-HCl (pH 8.0) containing 1 M sucrose, 1mM 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 then centrifuged twice at 9,700 x *g* for 20 min at 4˚C. The supernatant was diluted ten-fold with PBS. 3) Total cell lysate: Harvested cells were washed with PBS twice and resuspended in the same buffer at ca. 10 times the volume of the wet cell pellet. Cells were disrupted using an ultrasonic disruptor (UD200 TOMY) for 20 sec three

times at an output level of four. Unbroken cells were removed by centrifugation at 5,000 x g for 5 min. The supernatant was used as the cell lysate.

Immuno-affinity purification of Cdt holotoxin. Crude Cdt for immuno-affinity purification was prepared using an 80% saturated ammonium sulfate precipitation of the culture supernatant of *E. coli* carrying pTK3022. The precipitate after dialysis with wash buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.0), the crude CDT was applied to an affinity column where anti-CdtA antibody was coupled to CNBr-activated Sepharose 4B using the procedure described by the manufacturer (Amersham,). The CDT complex was eluted using elution buffer (0.2 M glycine-HCl, 0.2 M NaCl, pH 2.3) following immediate neutralization with 1/10 volume of 1 M Tris-HCl pH 8.0.

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Immunoprecipitaton and the pull-down assay. Immuno-precipitation using anti-serum against the Cdt components and pull-down assay were performed using the culture supernatant or the periplasmic fraction. For immuno-precipitation, ammonium sulfate precipitation of 3 l culture supernatant from *E. coli* carrying pTK3022 was prepared as described above. The precipitate was dissolved in 20 ml of PBS and dialyzed more than 4 h against 2 l PBS at 4 ˚C. The dialyzed sample was concentrated to ca. 1 ml using an Amicon centriprep YM-10 (Millipore) and subjected to pretreatment with or without SDS. The periplasmic fraction extracted from 500 ml of *E. coli* culture carrying pTK3022 was prepared as described above and concentrated to ca. 1 ml using an Amicon centriprep YM-10 (Millipore) and subjected to pretreatment with or without SDS. For SDS treatment, 1% SDS was added to the crude preparation of either culture supernatant or cell lysate, diluted 10-fold with PBS, and incubated with 2 µl of rabbit anti-CdtA, B, or C serum in an 1 ml sample for 1 h at 4˚C (38). Twenty microliters of Protein-A Sepharose beads (Amersham) were added and incubated further for 1 h. Beads were pelleted by centrifugation at 10,000 x *g* for 5 min, washed

three times in PBS, re-suspended in 70 µl of sample buffer containing 2 % SDS, 10 % ß-mercaptoethanol, 0.01 % bromophenol blue, 20 % glycerol, 120 mM Tris (pH 6.8), and boiled for 5 min. Beads were again pelleted by centrifugation; and the supernatant was analyzed using SDS-PAGE and immunoblot. Antisera against CdtA, CdtB, and CdtC were prepared as described by Sugai et al. (38). For the pull-down assay, culture supernatant and the periplasmic fraction of *E. coli* carrying pQE*cdtABC* were prepared as described above except pretreatment with SDS was performed. Five-hundred microliters of Ni-chelated agarose beads (Qiagen) were added to the sample solution and gently shaken for 1 h. The beads were recovered by centrifugation at 5,000 x *g* for 5 min and washed twice with 10 ml wash buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 20 mM imidazole). The sample was eluted with 2 ml elution buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 250 mM imidazole) by centrifugation at 5,000 x *g* for 5 min. The supernatant was used as the pull-down sample after dialysis against PBS and concentration using a Centricon 10 concentrator (Millipore, Bedford, MA).

Radio isotope labeling. Recombinant CdtA was radio labeled with [^{3}H]glycerol (specific activity 370 - 740 GBq/mmol, ARC) or [^{3}H]palmitate (specific activity 1.11 - 2.22 TBq/mmol, ARC, Tokyo, Japan) (25). Briefly, Cdt was expressed in 100 ml cultures of *E. coli* carrying pTK3022 or pUC*cdtA*^{C16G}*BC* by adding 1 mM IPTG at OD_{660}= ca. 0.5. At the same time, 148 KBq/ml (4 µCi/ml) of radio isotope was added to the medium and the culture was incubated for an additional 4 h (OD_{660} = ca. 0.9). After incubation and radio labeling, harvested cells was given a total membrane preparation as described elsewhere (25). The prepared membrane was dissolved in 100 µl PBS containing 1 % SDS and a 1 µl aliquot was withdrawn to measure the radio activity to determine the amount of total membrane using a liquid LSC5100 scintillation counter (AloKa) in 5 ml of scintillation cocktail Scintisol EX-H (DOJINDO,

Kumamoto, Japan). After the radio activities of the membranes were adjusted to ca. 250,000 ~ 300,000 cpm/100 µl of the [^{3}H]palmitate-labeled sample and 150,000 ~ 200,000 cpm/100 µl of the [^{3}H]glycerol-labeled sample, the samples (100 µl) were diluted 10 times with PBS and immunoprecipitation with anti-CdtA serum was performed. After immuno-precipitation (described above), all immuno-precipitated samples from 100 ml culture were electrophoresed by SDS-PAGE and the radio labeled proteins were visualized using fluorography with an Amplifier (Amersham).

Outer and inner membrane separation. *E.coli* outer and inner membrane separation was performed using a modification of the method described by Osborn (27). Briefly, *E. coli* cells carrying pTK3022 or pUCc*dtA*^{C16G}*BC* were harvested at OD_{660} = ca. 0.9 after induction with 1 mM IPTG for 4 h. Harvested cells were re-suspended 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 µl 10 mg/ml lysozyme (Sigma) for 2 min on ice. Spheroplasts were prepared by gradually adding 18 ml of 1.5 mM EDTA followed by ultrasonic disruption (UD-200, TOMY). Unbroken cells were removed by centrifugation at 5,000 x *g* for 5 min. Crude membranes (cell membrane fraction) were prepared using ultra-centrifugation at 100,000 x *g* for 35 min. Crude membranes were re-suspended in 0.5 ml 25 % sucrose and 5 mM EDTA. They were placed on a 5 % step gradient consisting of sucrose at 30 % (2.1 ml), 35 % (2.1 ml), 40 % (2.1 ml), 45 % (2.1 ml), 50 % (2.1 ml), and 55 % (0.5 ml) in 5 mM EDTA. The step gradient was ultra-centrifuged at 28,000 rpm for 20 h using a Beckman SW28.1 rotor. After centrifugation, fractionation (0.5 ml) was performed from the bottom of the tube under gravity. The protein concentration of each fraction was measured at OD_{595} using the Bio-Rad Protein Assay kit. Ten µl of each fraction was diluted into 800 µl with H_2O and mixed with 200 µl of the dye reagent provided in the kit. After incubation at room

temperature for 5 min, the absorbance was measured at 595 nm using an Amersham Ultraspec 100 Pro. Twenty microliters of each fraction was subjected to immunoblot for the detection of Cdt or Braun's lipoprotein. Braun's lipoprotein was used as the marker of the outer membrane fractions by immunoblotting with an antiserum provided by Dr. H. C. Wu, USUHS, Bethesda, MD (14). NADH oxidase was assayed in each fraction as the marker for the inner membrane fraction using the method described elsewhere (27).

DNA Techniques and plasmid construction. Routine DNA manipulations were performed using standard procedures. All restriction enzymes, T4 DNA ligase, and DNA polymerase were from Roche, Tokyo, Japan or New England BioLabs, Inc., Beverley, MA. Other materials and chemicals used were from commercial sources.

Site-directed mutagenesis of the sixteenth cysteine residue to glycine coded in the *cdtA* gene of pTK3022 was carried out using the overlap extension method (32). The primers used were: 5'-TTAGTGGCT**G**GTTCGTCA-3'; and 5'-TGACGAAC**C**AGCCACTAA-3'. The mutated DNA containing $cdtA^{C16G}BC$ (*cdtA* sixteenth cysteine changed to glycine) was subcloned into pUC19. In some experiments, the DNA fragment containing *cdtABC* in pTK3022 or the mutated DNA fragment containing $cdtA^{C16G}BC$ was cloned into pMW219 (Nippon gene, Osaka, Japan) which is a plasmid with a low copy number in *E. coli* (3).

Other procedures. SDS-PAGE and Western blot (Immunoblot) were carried out as described previously (38). Immuno-detection was carried out using a Renaissance 4CN plus (Dupont-NEN). The N-terminal sequence was performed using a Model 49X Procise (Applied Biosystems) after removing the Coomassie-stained bands and transferring to a PVDF membrane. ESI-Mass spectrometry was performed using an

Applied Biosystems/MDS-Sciex mass analysis following the manufacture's instructions.

RESULTS

CdtA is a lipoprotein. The bacterial CdtA lipoproteins were found to be a covalently linked lipid to the cysteine residue at the N-terminus. These lipoproteins presumably share a common biosynthetic pathway to generate *N*-acyl-diacylglycerylcysteine after cleavage of the N-terminal signal peptide which carries a consensus sequence, the so-called "lipobox" (-Leu-X-Y-Cys-) (40). We previously showed *A. actinomycetemcomitans* CdtA possesses the lipid binding consensus lipobox (-Leu-Val-Ala-Cys-) in the N-terminal signal sequence suggesting *A. actinomycetemcomitans* CdtA is a lipoprotein (38). To determine if the lipobox motif is present in the signal sequence of Cdt in other species, we examined published amino acid sequences of Cdt homologues deposited in the database. As shown in Table 2, possible lipobox sequences were found in the signal sequence of either CdtA or CdtC in all investigated species. This suggests the lipobox signature is ubiquitous in either CdtA or CdtC among a variety of Cdt-producing bacteria. We then determined if *A. actinomycetemcomitans* CdtA is a lipoprotein *in vivo*. *E. coli* carrying pTK3022 was incubated with [^{3}H]palmitate or [^{3}H]glycerol to metabolically label proteins covalently linked with lipid; and the membrane fraction was immuno-precipitated using anti-CdtA serum. In addition, a plasmid encoding *cdtABC* whose sixteenth cysteine residue of CdtA was mutated to glycine was constructed and the transformant *E. coli* carrying pUC*cdtA*^{C16G}*BC* was treated with the same procedures. As shown in Fig. 1A, B, a radio labeled band with a molecular mass of 23 kDa (indicated by an arrow) was observed in the total membrane of *E.coli* carrying pTK3022. Whereas, this band was

missing in *E. coli* carrying the cysteine deficient pUC*cdtA*^{C16G}BC. This suggests the lipid is covalent linked to the sixteenth cysteine of CdtA. To further demonstrate CdtA is a lipoprotein, we examined the accumulation of a precursor form of CdtA in cells treated using globomycin, a specific inhibitor of the signal peptidase II (14, 15). Globomycin-treated bacteria accumulate the glyceride-modified precursors of lipoproteins (14). *E. coli* carrying pUC*cdtABC* was incubated with [^{3}H]palmitate in the presence or absence of globomycin, and the membrane fraction was probed for CdtA using immuno-precipitation. As shown in Fig. 1D, globomycin induced the appearance of an additional radio labeled band with a slightly higher molecular mass in SDS-PAGE gels indicating the accumulation of the glyceride-modified precursor form of CdtA. These results clearly indicate CdtA is a lipoprotein.

Lipid-modified CdtA localizes in the outer membrane. Since one of the important features of a lipoprotein is membrane localization due to lipid insertion in the membrane lipid bilayer, we next attempted to determine if lipid-modified CdtA localized in the inner or outer membrane. We used an *E. coli* membrane fractionation method to separate *E. coli* carrying pTK3022 membrane by spheroplast formation and subsequent sucrose density gradient ultra-centrifugation (27). As shown in Fig. 2A, membrane fractionation revealed authentic fractionation patterns and generated two protein peaks as expected. Presence of Braun's lipoprotein in the first peak demonstrated that the first peak corresponds to the outer membrane. The second peak was NADH oxidase positive showing it to be the inner membrane. Immunoblot shows CdtA was present only in the outer membrane fraction. This result agrees with the +2 rule of the lipobox serine residue which is located next to the sixteenth cysteine residue in the outer membrane (35). At the same time, we prepared the membrane fractions from *E. coli* carrying pUC*cdtA*^{C16G}*BC*. The mutated CdtA^{C16G} was found mainly in

the inner membrane fractions suggesting that the sixteenth cysteine residue is important for localization in the outer membrane (Fig. 2B). Since the three Cdt components are shown to form a tripartite complex in a stoichiometry of 1:1:1 in the culture supernatant (19, 22), we attempted to determine if the membrane-bound CdtA was associated with either CdtB and/or CdtC. Immunoblots of the membrane fractions of *E. coli* carrying pTK3022 show CdtB and CdtC in the outer membrane (Fig. 2A).

Cdt complexes in the periplasm consists of CdtA, CdtB, and CdtC. We next determined if the Cdt components were present as a complex in the periplasmic space. Immuno-precipitation and Western blot of the crude preparation of periplasm were performed. The crude preparation of the periplasm was treated with or without SDS (final 1%), diluted ten times, and incubated with anti-Cdt serum. The immune complex was recovered using Protein A-Sepharose 4B beads and analyzed using immunoblot. In the SDS-treated sample, anti-CdtA serum immuno-precipitated CdtA but not CdtB or CdtC. Conversely, in the sample without SDS-treatment, anti-CdtA serum immuno-precipitated not only CdtA but also CdtB and CdtC. An extra-band with a molecular mass of 18 – 19 kDa reacting with anti-CdtA serum was observed. This band was named CdtA'. Anti-CdtC serum also immuno-precipitated all three components in the absence of pretreatment with 1% SDS. Because the bands smeared at ca. 25 dDa (possilby the immunoglobulin light chain) and obscured the CdtA bands in the immuno-precipitated samples, we prepared a crude fraction of the periplasm of *E. coli* carrying pQE*cdtABC* and attempted to pull down the associated proteins with 6x histidine-tagged CdtC using Ni-beads. Figure 3C shows the pull-down of CdtA, CdtA', CdtB and CdtC from the periplasm. This indicates Cdt is present as a complex in the periplasm and the complex consists of CdtA, CdtB, and CdtC with a very small amount of CdtA'. In order to determine if CdtA in the periplasm is a lipid-modified

form, *E. coli* carrying pTK3022 was incubated with [^{3}H]palmitate and the periplasmic fraction was isolated. The fraction was subjected to immuno-precipitation using anti-CdtA serum. The precipitate was separated in SDS-PAGE gel followed by fluorography. As shown in Fig.1C, a faint radio labeled band corresponding to 23 kDa was observed. This indicates that at least part of the CdtA in the complex in periplasm is in the lipid-modified form.

CdtA in the complex goes through another processing step to form the mature form. The immuno-precipitation and pull-down experiments were also performed using the culture media supernatant. As shown in Fig. 3B and D, Western blot analysis demonstrated a difference in the ratio of CdtA and CdtA' in the immuno-precipitated or pull-down sample between the culture supernatant and the periplasmic fraction. Cdt holotoxin in culture supernatant contained almost solely CdtA'. To determine the molecular nature of the CdtA', a component of Cdt complex principally found in culture supernatant, we prepared a Sepharose 6B (Amersham) conjugated with affinity-purified anti-CdtA-antibody and purified Cdt holotoxin from the culture supernatant of *E. coli* carrying pTK3022. The immuno-affinity purified Cdt holotoxin recovered from the column showed a specific activity of 1.13×10^6 CD_{50}/mg. As shown in Fig. 4, SDS-PAGE analysis of the eluted fraction demonstrated the presence of three major bands stained with Coomassie brilliant blue. The calculated molecular mass of the proteins from the PAGE were 30, 20, and 18 - 19 kDa. N-terminal amino acid sequence of each protein band was determined using a PROCITE gas phase protein sequencer. The amino acid sequence of the 30 kDa (1) and 20 kDa (2) protein were NLSDFKVATW and ESNPDPTTYP, respectively. Two amino acid sequences, ASSMPLNLLS (3-1) and LLSSSKNGQV (3-2), were obtained from the broader band of 18 – 19 kDa. Comparison with the deduced amino acid

sequences shows the 30 kDa protein corresponds to CdtB and 20 kDa band to CdtC without the predicted signal peptides (Fig. 4A). The broader band of 18 – 19 kDa was a composite of the N-terminally processed fragments of the lipid-modified CdtA. The immunopurified complex was further analyzed using ESI-MS. Four major peaks were obtained with masses of 18,912.4, 18,211.6, 28,886.6 and 18,410.8. This was in agreement with the calculated molecular masses of CdtA'-1 at 18,915; CdtA'-2 at 18,215; CDTB at 28,880; and CdtC at 18,622 from the predicted amino acid sequence of the mature Cdt components. These results indicate that CdtA'-1 and CdtA'-2 are N-terminally truncated forms beginning at the 52^{nd} alanine and 58^{th} leucine, respectively.

The above results show *A. actinomycetemcomitans* Cdt is present as a complex of CdtA or CdtA', CdtB, and CdtC in the periplasm and a complex of CdtA', CdtB, and CdtC in the culture supernatant of *E. coli* carrying pTK3200. We therefore characterized the constituents of the Cdt complex in *A. actinomycetemcomitans*. As shown in Fig. 4B, immuno-precipitated Cdt from *A. actinomycetemcomitans* cell lysate consists of CdtA, CdtB, and CdtC, whereas that from *A. actinomycetemcomitans* culture supernatant principally was CdtA', CdtB, and CdtC. These data strongly suggests similar processing of CdtA takes place not only in *E. coli* but also in *A. actinomycetemcomitans*.

Lipid modification of CDTA is important for secretion of the CDT complex. We next determined the biological importance of the lipid-modification of CdtA. We used a low copy number plasmid, pMW219 to express the Cdt holotoxin to mimick the expression of *CdtABC* genes in *A. actinomycetemcomitans*. We compared Cdt activities in the culture supernatant and in the sonic lysate of *E. coli* carrying pMW*cdtABC* and in *E. coli* carrying pMW*cdtA*^{C16G}*BC*. As shown in Fig. 5, the Cdt

activity in the culture supernatant of *E. coli* carrying pMW*cdtA*^{C16G}*BC* was significantly lower when compared to the strain producing the wild type Cdt. Whereas this significant change was not observed in the Cdt activity in the cell lysate.

DISCUSSION

After cloning of the *cdt* genes from many microorganisms, several reports have shown CdtA or CdtC components possess a putative lipid modification motif (28, 29, 38). However no experiments have been conducted to determine the exact lipid modification of CdtA or CdtC. With *A. actinomycetemcomitans*, the lipobox was observed in the *CdtA*. In a previous study, we demonstrated every Cdt component is present not only in the periplasm but also in the culture supernatant of *A. actinomycetemcomitans* as well as in an *E. coli* carrying the plasmid containing *A. actinomycetemcomitans CdtABC* (23). These results show Cdt was extracellularly secreted in both organisms and that the *E. coli* system worked well in mimicking the production of Cdt in *A. actinomycetemcomitans*. Western analysis shows an anti-CdtA-reactive band with a molecular mass lower than CdtA in the supernatant fraction of both microorganisms. This suggets CdtA undergoes proteolytic processing during biogenesis of the Cdt holotoxin. To analyze the lipid modification using radiolabeling to characterize biogenesis of Cdt holotoxin, fairly large amounts of toxin production are necessary. We therefore used *E. coli* carrying *cdtABC* genes on a multicopy plasmid to characterize the *cdt* gene products and to extrapolate the biogenesis of Cdt holotoxin in *A. actinomycetemcomitans*. This is the first report to demonstrate the incorporation of palmitate or glycerol into CdtA that indicates CdtA is a lipoprotein. Susceptibility of the signal sequence-processing of CdtA to globomycin further supports this. Lipid-modified CdtA is present in either the outer membrane or

in the periplasm as a complex with CdtB and CdtC. However, how the lipid-modified CdtA is released into the periplasm remains to be determined. A possibility is the assembly with CdtB and CdtC enforces the detachment of CdtA from the outer membrane. This requires confirmation. Purification of the Cdt complex from the culture supernatant using anti-CdtA antibody affinity chromatography shows CdtA' in the complex is an N-terminally truncated form of CdtA. Together with the observation that CdtA' was found in the immuno-precipitated sample of the periplasm fraction indicates the lipid-modified CdtA undergoes further processing of the N-terminus in the periplasm and the complex with processed CdtA (CdtA') will be secreted from the periplasm to the culture supernatant. This agrees with previous reports *A. actinomycetemcomitans* CdtA exists in two forms in crude extracts at 25 and 18 kDa (36).

Figure 6 shows our current model for the biogenesis of the Cdt complex based on the observations obtained in this study. We suggest the unmodified CdtA, immature CdtB and immature CdtC are secreted into the periplasmic space through a *sec*-dependent secretion pathway (data not shown). After translocation to the inner membrane, immature CdtB and CdtC undergo processing by signal peptidase I. Unmodified CdtA then undergoes processing by lipoprotein-specific signal peptidase II followed by lipid-modification. After the amide-linked fatty acid modification at the N-terminal cysteine, lipid-modified CdtA is transported to the outer membrane where the lipid-modified CdtA, CdtB, and CdtC forms a complex and subsequently is released into the periplasm. Processing of N-terminal ca. 40 amino acids of CdtA by an unidentified protease(s) takes place in the periplasm, and the resulting mature holotoxin is secreted into the culture medium by an unknown mechanism. The significant decrease of Cdt activity in the culture supernatant of *E. coli* carrying pMW*cdtA*^{C16G}*BC*

strongly suggests the inability of lipid modification of CdtA affects secretion of Cdt holotoxin into the culture supernatant. Membrane anchoring of lipid-modified CdtA may be important for the efficient formation of the Cdt complex or for efficient secretion. Further studies regarding the *in vivo* kinetics of the complex formation and subsequent secretion are necessary to understand the biological importance of lipid-modification of the CdtA in *A. actinomycetemcomitans*.

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Figure legends

Fig. 1. **Lipid modification of CDTA.** Bacterial cells were radio labeled with either [^{3}H]glycerol or [^{3}H]palmitate. The cell membrane and the periplasmic fractions were solubilized with 1% SDS-containing buffer. A 1:10 dilution of the SDS preparation with PBS was made and the CdtA was immuno-precipitated using anti-CdtA serum and purified with protein A Sepharose. The immuno-precipitated protein was separated by SDS-PAGE followed by fluorography. Panel A, [^{3}H]glycerol-labeled membrane fraction; Panel B, [^{3}H]palmitate-labeled membrane; Panel C, [^{3}H]palmitate-labeled periplasmic fraction. Lane 1: *E. coli* carrying pTK3022, 2: *E. coli* carrying pUC*cdtA*^{C16G}*BC*. 3: *E. coli* carrying vector only (only in panel A). Arrow indicates the lipid-modified CdtA. Panel D, effect of globomycin on the processing of CDTA. Various concentrations of globomycin, a signal peptidase II specific inhibitor, was added to the culture 30 min before labeling with [^{3}H]palmitate. After membrane preparation, the radio labeled bands of lipid-modified prolipoprotein, diacylglyceryl (DG-) proCdtA where the signal sequence remains uncleaved, and mature lipoprotein lipoCdtA were ressolved by SDS-PAGE followed by fluorography.

Fig. 2. **CdtA in the outer membrane.** Panel A, Outer and inner membrane separation of *E. coli* carrying pTK3022 (A) or pUC*cdtA*^{C16G}*BC* (B) was performed after spheroplast isolation using sucrose-density gradient separation. After fractionation (0.5 ml / tube) from the bottom of the tube, each fraction was assayed for protein concentration (line) using the BioRad protein assay kit. The OD_{595} represents relative protein concentrations. NADH oxidase activity (bars) shows the inner membrane fractions. Immunoblots of each fractions are shown using anti-CdtA, anti-CdtB, anti-CdtC or anti-Braun's lipoprotein serum. The anti-Braun's lipoprotein serum

shows the outer membrane fractions.

Fig.3. **Immunoprecipitation and the pull-down assay for the Cdt holotoxin.** Crude Cdt was prepared from either the periplasmic space (A) or the culture supernatant (B) of *E. coli* carrying pTK3022. Cdt components were immuno-precipitated with anti-CdtA or anti-CdtC in the presence or absence of SDS-pretreatment at a concentration of 1%. Immuno-precipitated samples were analyzed using SDS-PAGE and immunoblot using rabbit anti-CdtA, anti-CdtB or anti-CdtC. The smeared bands at ca. 25 kDa may be rabbit immunoglobulin light chains.
Similarly, crude Cdt was prepared from either periplasmic space (C) or culture supernatant (D) of *E. coli* carrying pQE*cdtABC*. Cdt components were pulled down with Ni-chelated beads using 6x his-tagged CdtC from *E. coli* carrying pQE*cdtABC*. Pull down samples were subjected to SDS-PAGE and immunoblot using rabbit anti-CdtA, anti-CdtB or anti-CdtC.

Fig.4. **Immunoaffinity purification of the Cdt complex.** Crude Cdt was prepared using 80% saturated ammonium sulfate precipitation of the culture supernatant of *E. coli* carrying pTK3022. After dialysis with wash buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8), crude Cdt was applied to an affinity column where anti-CdtA antibody was coupled to CNBr-activated Sepharose 4B. The Cdt complex was eluted with elution buffer (0.2 M glycine-HCl, 0.2 M NaCl, pH 2.3) followed by immediate neutralization with a 1/10 volume of 1 M Tris-HCl pH 8.0. The complex was analyzed using SDS-PAGE with Coomassie brilliant Blue staining (A). Detected N-terminal amino acid sequences of each Coomassie-stained band are shown (A, right). B, immunoaffinity purification of Cdt complex from *A. actinomycetemcomitans* Y4 culture

supernatant. Crude Cdt was prepared by 80% saturated ammonium sulfate precipitation of the culture supernatant of *A. actinomycetemcomitans* Y4. Immuno-affinity purification using anti-CdtA affinity column and immunoblot was performed as described above. Lanes 1, *A. actinomycetemcomitans* Y4 total cell lysate; 2, immuno-purified sample from *A. actinomycetemcomitans* Y4 culture supernatant; 3, immuno-purified sample from culture supernatant of *E. coli* carrying pTK3022.

Fig. 5. **Lipid modification and cytodistending activity.**
Wild type and mutated Cdt holotoxin were produced from *E. coli* carrying pMW*cdtABC* and pMW*cdtA*^{C16G}*BC*, respectively. Both cells cultured from the same OD_{660} = 0.05 and incubated using the same conditions with vigorous shaking. Both samples were harvested at logarithmic phase (OD_{660} = 0.5); then the culture supernatant (A) and cell pellet were prepared. Cells were resuspended in PBS at pH7.3 and ultrasonically disrupted (cell lysate, B). Both supernatant (0.5 µg/ 100 µl) and cell lysate (100 µg/ 100 µl) fractions were sterilized using 0.22 µm-pore filter and titrated for cytodistending activity (CD_{50}/mg) on HeLa cells using the serial dilution described in the MATERIALS AND METHODS.

Fig. 6. Hypothetical model of Cdt complex formation and secretion. Immature CdtA, CdtB, and CdtC are translated from the *CdtA*, *CdtB*, and *CdtC* genes and secreted into the periplasmic space in a *sec*-dependent general secretion pathway using their N-terminal signal sequences. During passing through the inner membrane, the signal sequences are cleaved and immature CdtB (imCdtB), and immature CdtC (imCdtC) become mature forms using the truncating signal peptidase I. Unmodified CdtA

(unCdtA) is cleaved at its signal sequence by signal peptidase II and modified with lipid. The lipid-modified CdtA (lipoCdtA) is carried to the outer membrane using the serine residue next to lipid-modified cysteine residue. LipoCdtA forms a complex with CdtB and CdtC in the outer membrane and periplasmic space. The lipid moiety of CdtA may be hidden by CdtA itself, or by CdtB and CdtC in the periplasm. Finally an unknown protease(s) cleaves N-terminal ca. 40 amino acids of the lipoCdtA and then the complex is secreted into the culture medium. Molecular sizes and structures of the Cdt complex are arbitrarily estimated.

Table 1. Strains used in this study

Strain		Plasmid	Character	Reference
A.actinomycetemcomitans Y4			standard strain	
E.coli	XL1 blue	pTK3022	*cdtABC* on pUC19 (*Sma* I-*Eco* RI)	Sugai, 199
	M15	pQE*cdtABC*	*cdtABC* on pQE60 (*Nco* I-*Bgl* II)	Ohara, 200
	XL1 blue	pUC*cdtA* ^{C16G} *BC*	*cdtA* ^{C16G} *BC* on pUC19 (*Sma* I-*Eco* RI)	This study
	XL2 blue	pMW*cdtABC*	*cdtABC* on pMW219 (*EcoRI* -*Eco* RI)	This study
	XL2 blue	pMW*cdtA* ^{C16G} *BC*	*cdtA* ^{C16G} *BC* on pMW219 (*EcoRI* -*Eco* RI)	This study

Table 2. Possible lipobox consensus sequence in various CDTs

Strains		Subunits	Possible signal sequence (*lipobox*)	Gene accessio
A. actinomycetemco		A	$_{+1}$MKKFLPGLLLMG***LVAC***$_{+17}$S	AB011405
H. ducreyi		A	$_{+1}$MKKFLPSLLLMG***SVAC***$_{+17}$S	U53215
C. jejuni		A	$_{+1}$MQKIIVFILCCFMTFF***LYAC***$_{+21}$S	U51121
H. hepaticus		A	$_{+1}$MRLLFFLLITLL***FAAC***$_{+17}$S	AF163667
E. coli	I	A	$_{+1}$MDKKLIAFLCTLI***ITGC***$_{+18}$S	U03293
		C	$_{+1}$MKTVIVFFVLL***LTGC***$_{+16}$A	
	II	A	$_{+1}$MANKRTPIFIAGILIPIL***LNGC***$_{+23}$S	U04208
		C	$_{+1}$MKKLAIVFTMLL***IAGC***$_{+17}$S	
	III	A	$_{+1}$MTNKCTSILIVGILIPIL***LNGC***$_{+23}$S	U89305
		C	$_{+1}$MKRLIIIVTMLL***IAGC***$_{+17}$S	

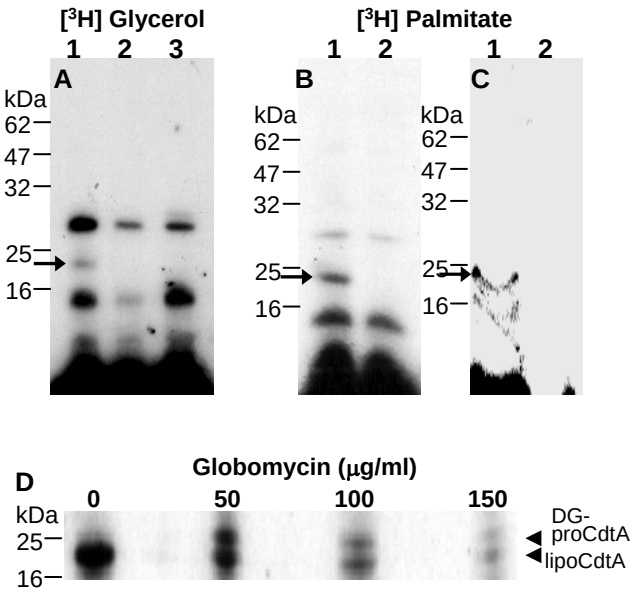

Fig.1
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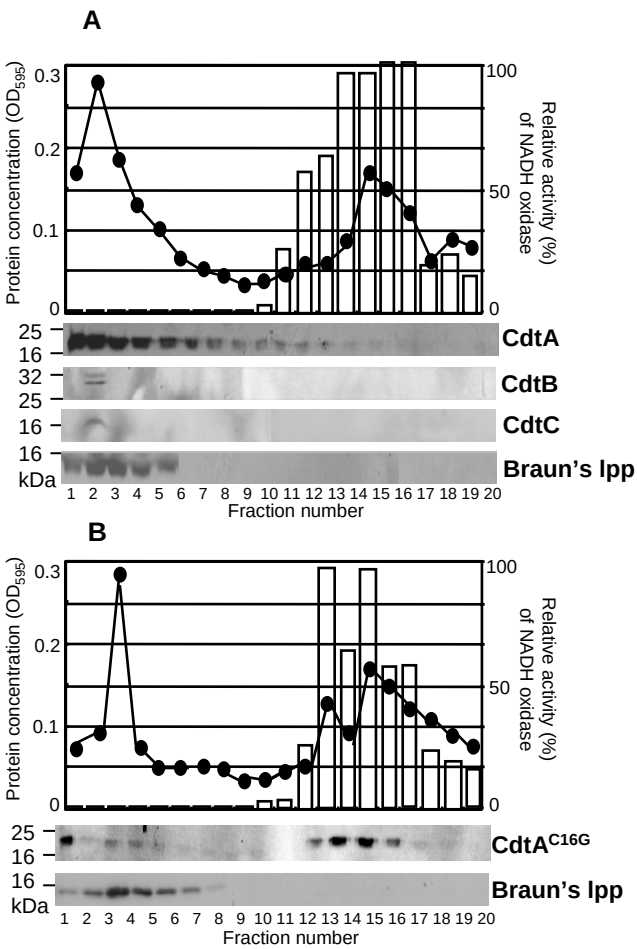

Fig.2
Ueno, Y., et al

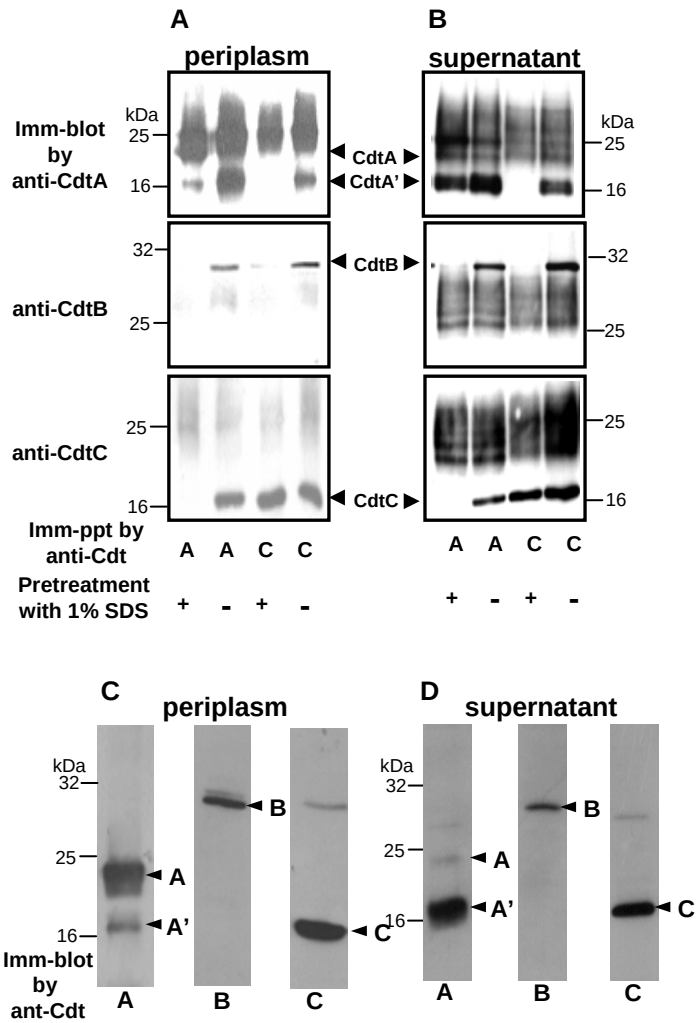

Fig.3
Ueno, Y., et al

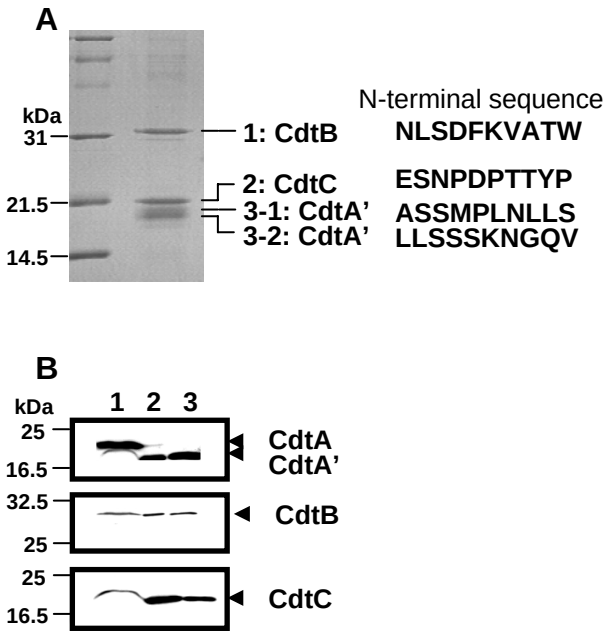

Fig.4
Ueno, Y., et al

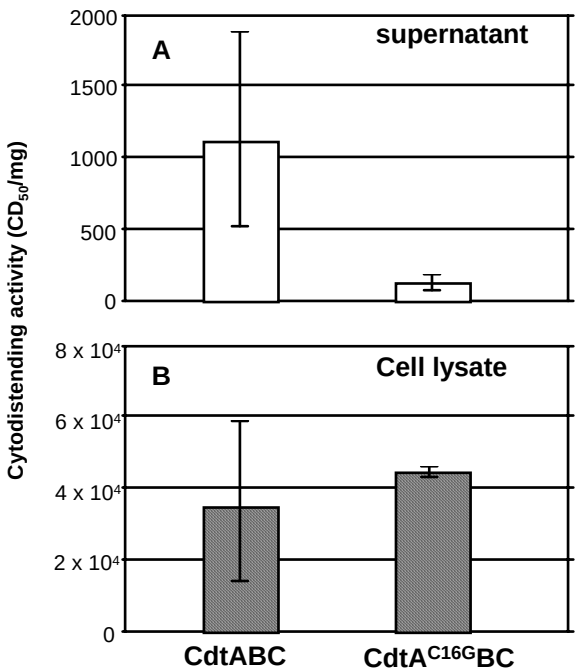

Fig.5
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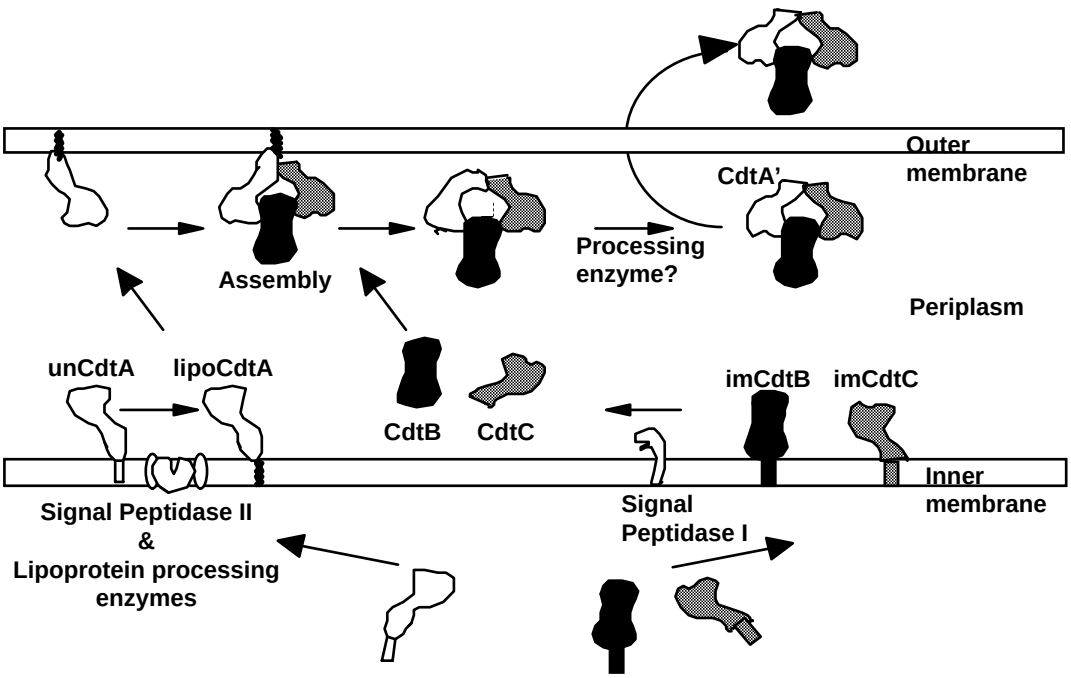

Fig.6
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