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Cloning and protein expression of Actinobacillus actinomycetemcomitans cytolethal distending toxin subunit CdtA

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I. INTRODUCTION

Periodontitis is a persistent bacterial infection, which causes chronic inflammation that leads to the destruction of the tooth supporting tissues. A wide variety of bacterial species are implicated in the pathogenesis of the disease. One of these is the Gram-negative facultative anaerobe *Actinobacillus. actinomycetemcomitans*, a species highly associated with localized aggressive periodontitis. This form of the disease occurring in adolescents and young adults is characterized by rapid and severe loss of supporting alveolar bone and connective tissue attachment[1]. It remains unclear that the general pathogenic mechanisms and immune involvement in periodontitis, in particular the bacterial virulence factors responsible for the proposed immunosuppression in this disease. *A. actinomycetemcomitansis* occasionally responsible for non-oral infections including endocarditis, bacteremia, pericarditis, septicemia, pneumonia, infectious arthritis, osteomyelitis, synovitis, skin infections, urinary tract infections, and abscesses[2]. It has been estimated that approximately 0.6% of cases of infective endocarditis are caused by *A. actinomycetemcomitans*[3]. Studies suggest that bacterial virulence factors act to impair host mechanisms and play significant roles in infectious diseases associated with *A. actinomycetemcomitans*[4-6]. These virulence factors included leukotoxin, lipopolysaccharide(LPS), and cytotoxin-cytolethal distending toxin(CDT).

CDT was first documented as a toxin oc-

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curring in Escherichia coli strains by Johnson and Lior in 1987[15,26,27]. CDT has been identified and cloned from *A. actinomycetemcomitans*[7-9], and various other pathogenic bacteria[10-18]. The term CDT reflected the unique properties of this protein causing progressive cell distension and cytotoxicity to cultured cells such as CHO, HeLa, and Hep-2.

Another unique phenotype of the CDT-intoxicated cells is cell cycle arrest at the transition from G2 to M phase. *A. actinomycetemcomitans* is the only oral bacterium known to produce a cytolethal distending toxin(CDT), which causes growth arrest in mammalian cells[19-22].

CDT is prevalent in *A. actinomycetemcomitans strains*. Ahmed et al.(2001) found that 43 of 50 strains from periodontitis patients contained all three cdt genes and expressed CDT activity. In another study, PCR of subgingival plaque samples revealed that 13 of 106 diseased sites in 146 patients with aggressive and chronic periodontitis contained *A. actinomycetemcomitans* expressing all three cdt genes[24]. Fabris et al.(2002) reported that 39 of 40 *A. actinomycetemcomitans* isolates from a mix of healthy and periodontal diseased subjects expressed activity that caused the distension of CHO cells.

The genes of cdt have been isolated as *cdtA*, *-B*, and *-C*, tandemly located in the cdt locus. Most recent studies indicate that all three components, *cdtA*, *-B*, and *-C*, are required for full activity[28-32]. Pull-down assay revealed that the active CDT holotoxin is a heterotrimer, consisting of CdtB as the enzymatically active subunit, and CdtA and CdtC which mediate the delivery of CdtB into host cells[28-31].

A study of Lee et al.(2003) with *C. jejuni* showed both CdtA and CdtC, but not CdtB, were able to bind individually to the surface of HeLa cells using a common receptor[33]. Mao and DiRienzo(2002) demonstrated that only CdtA was able to bind to the surface of CHO cells. Following internalization conversely, no cytotoxic effects were observed. On the contrary, the introduction of CdtB or CdtC into the cells did cause cell distention and eventually cell death. This study urges one to conclude only CdtA is responsible for the attachment to the cell and the CdtC subunit assists CdtB in the cytolethal activity of the holotoxin[34].

The precise role of the CdtA protein in the mechanism of action of the holotoxin is unknown at the present time. The ultimate goal of this study is to develop an edible vaccine against *A. actinomycetemcomitans*, causative agent for LAP. As an initial step, I cloned cdtA gene from *A. actinomycetemcomitans* and expressed CdtA protein.

II. MATERIALS AND METHODS

1. Materials

All chemicals and plastic wares were purchased from Sigma (St. Louis, MO) and Falcon Labware (Frankline Lakes, NJ),

respectively. Oligonucleotides were ordered from COSMO. T vector and Tag polymerase was supplied from Promega(USA). Restriction enzymes were obtained from BioLabs (New England).

2. Bacterial culture

A. actinomycetemcomitans Y4 was grown in Luria-Bertani(LB) broth medium for 24 h at 37℃ in an atmosphere of 5% CO_2 in air and 200㎕ of overnight culture was transferred into 5㎖ of a fresh medium, leaving for further incubation.

3. Preparation of genomic DNA

About 1 ㎖ of an overnight culture of *A. actinomycetemcomitans* Y4 was collected and added to a 1.5 ㎖ microcentrifuge tube. The tube was centrifuged at 13,000~16,000 ×g for 2 minutes to pellet the cells. Remove the supernatant. After addition 600 ㎕ of Nucleus Lysis Solution, gently pipeted until the cell are resuspended. The mixture was further incubated at 80℃ for 5 minutes to lyse the cells; then cooled to room temperature. After addition 3 ㎕ of RNase Solution to the cell lysate, the sample was again incubated at 37℃ for 15~60 minutes then cooling to room temperature. Finally added 200 ㎕of Protein Precipitation Solution was added with vigorouse vortexing. The vortexed sample was incubated on ice for 5 minutes, then centrifuged at 13,000~16,000 ×g for 3 minutes.

4. PCR amplification of cdtA gene

The genomic DNA was used as templates for amplifying *cdtA* gene in 50㎕of PCR reaction containing Taq polymerase, dNTP, and oligonucleotides, primers, 10×PCR buffer. The primers specific for cdtA gene were designed and synthesized as described in Table 1. The genomic DNA was denatured by 5 min incubation at 94℃ and then target genes were amplified for 30 cycles of PCR reaction (1 min 94℃, 1 min 63℃, 1 min 72℃). The PCR product was then treated for 10 min at 72℃ and stored at 4℃.

5. Cloning of *cdtA* gene

PCR products were ligated into pGM-T vector following manufacture's protocol and the ligation mixtures were transformed into competent *E. coli* Top10 cells. The resulting antibiotics resistant bacteria were selected from agar plates and further screened by

Table 1. List of oligonucleotides used to amplify cdtA genes. The underlined sequences are the locations of restriction enzyme sites.

	Sequence
cdtA (F)	ATG CGG ATC CAT GAG TGA CTA TTC TCA G
cdtA (R)	ATG CCT GCA GTT AAT TAA CCG CTG TTG C

checking the plasmid from each bacterial colony. First, plasmids were compared in size with that of control vector in agarose gels to reveal the presence of any insert DNA. Second, digestions of plasmids with restriction enzymes were performed to further prove the presence of target genes. The plasmids containing expected insert size were then sequenced to confirm the integrity of target gene. The DNA sequencing was performed by Xenotech (Korea).

6. Sequence analysis of cdtA gene from *A. actinomycetemcomitans* (Y4)

The DNA sequence of cloned genes was analyzed by comparing with the previously known sequences. For cdtA sequence analysis, the known sequence from *A. actinomycetemcomitans* (*Y4*)(GenBank GI9955924) was used as standard. The internet site (www.ualberta.ca) were used to generate sequence alignment between two genes.

7. Expression of recombinant CdtA protein in bacterial expression system

In order to express cloned genes in *E. coil* system, gene fragments of *cdtA* were subcloned to pRSET expression vector. BamHI and PstI were used to transfer *cdtA* gene. The ligation mixtures were transformed in TOP10 host cell. The ampicillin resistant bacteria were selected from LB agar and transformed BL21(DE3) cell. Transformed BL21(DE3) bacterial colonies were inoculated and grown overnight at 37℃ in LB agar containing 100㎍/㎖ of ampicillin. The cells will be further grown for 4h in the presence of 1mM IPTG to induce expression of the fusion protein.

8. Isolation of recombinant CdtA protein

The cells were obtained by centrifugation at 6000rpm for 10minutes. The cells were suspended in phosphate buffer saline (PBS). The suspension was sonicated for three 30seconds bursts on ice. The sonicated samples were centrifuged at 13,000rpm for 10 minutes. The supernatant fluid was then passed through a 0.45 micron filter to obtain the soluble fractions. Cell pellets were washed with PBS containing 0.01% Triton X-100 and resuspended in PBS to obtain the inclusion body.

9. Analysis of CdtA protein (SDS-PAGE, Western blot)

The factions containing recombinant protein (6×His-tagged protein) were identified by SDS-PAGE. Bacteria collected from 1ml of IPTG-induced culture were mixed with 100 ㎕ of gel loading buffer and heated in a boiling water bath for 5min. Samples were applied to a 10~20% polyacrylamide gel (25 ㎍ of protein per lane). After electrophoresis, protein bands were transferred to a nitrocellulose membrane. The membrane was

blocked with 3% BSA in TBS buffer (20mM Tris-HCl, pH 7.6, 0.8% NaCl) for 1h at room temperature with shaking. The blocked membrane was added Ni-NTA tagged with AP (alkaline phosphate) and then washed in TBS buffer. The membrane was added BCIP/NBT to induce developing reaction and incubated in the dark. The membrane was rinsed with dH_2O to stop the developing reaction.

III. RESULTS

1. PCR product of cdtA gene

The genomic DNA from *A. actinomycetemcomitans* Y4 was isolated and used as templates for amplifying cdtA genes. The primer specific for cdtA gene was separately designed based on the published sequences. PCR was performed yielding 0.6 kb cdtA (Figure 1).

2. Cloning into T-vector of cdtA

PCR products were ligated into T vector, yielding T vector cloned with *cdtA*. The cloned T vectors were transformed into an *E. coli strain*, Top 10. Plasmids were prepared from antibiotics-resistant colonies and separated in agarose gel (Figure 2). *In T-cdtA* agarose gel, the 4th lane band is lower than the others because it does not include insert DNA.

3. Sequence analysis of cloned genes

After checking the presence of any insert in the ligated plasmid, we confirmed the integrity of cloned cdtA sequence by nucleotide sequencing. For cdtA sequence analysis, the corresponding sequence from *A. actinomycetemcomitans* Y4 was compared with that of the previously-cloned gene. The rep-

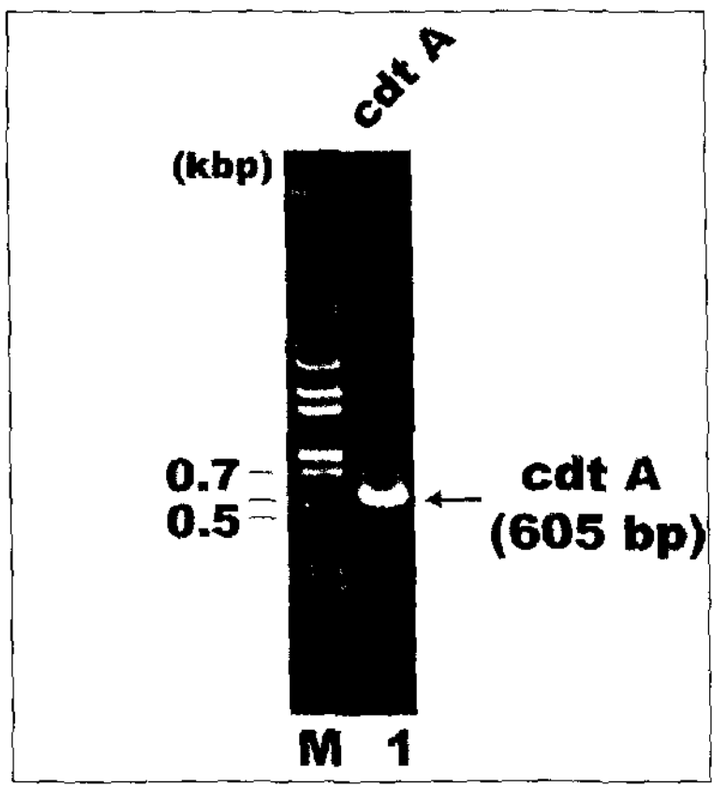

Figure 1. PCR amplification of *A. actinomycetemcomitans* Y4 genes encoding cdtA. The genes for cdtA were amplified by PCR using the genomic DNA linearized by restriction enzyme digestion.

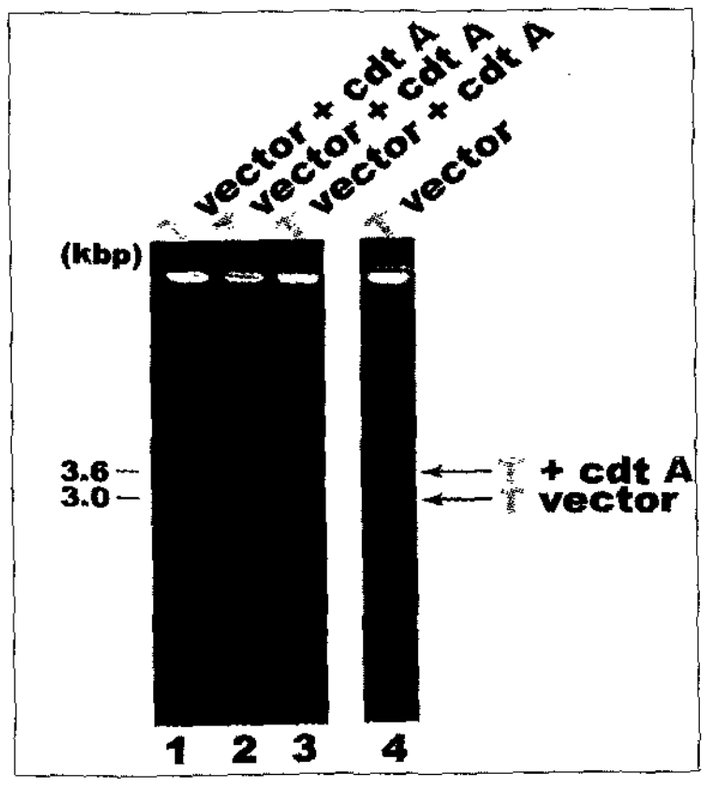

Figure 2. Screening of plasmids cloned with PCR-amplified cdtA genes. The genes encoding *cdtA* were cloned into T vector and bacterial transformants were screened for the presence of insert DNA.

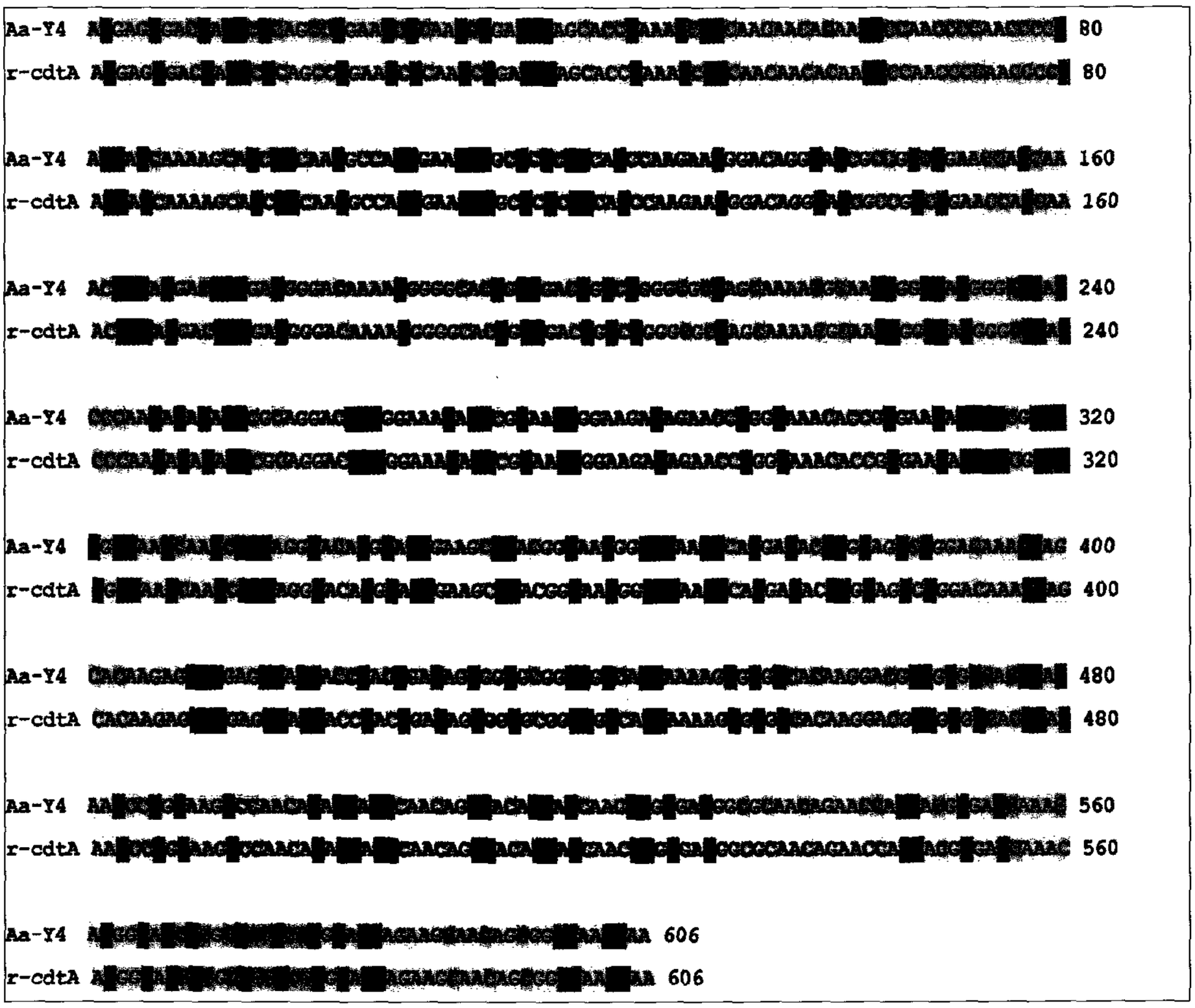

Figure 3. Sequence alignment between the cloned *cdtA* gene and the corresponding region of the reported *cdtA* gene. The sequence of the cloned *cdtA* gene was compared to that of known *cdtA* gene.

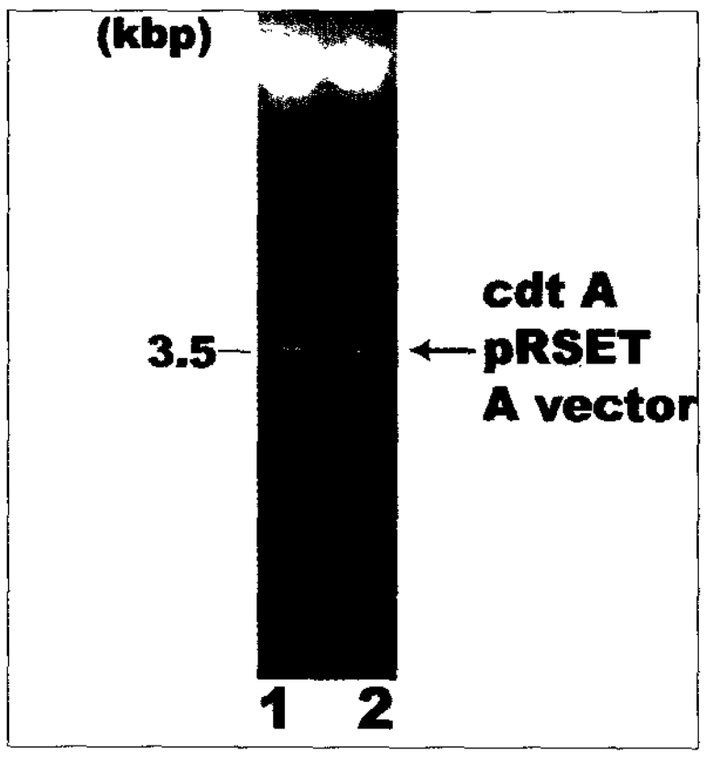

Figure 4. Screening of plasmids of cdtA cloned in pRSET vector. The pRSET-cdtA plasmid was transformed in BL21(DE3) bacterial strains, and their transformants were screened for the presence of recombinant expression vector.

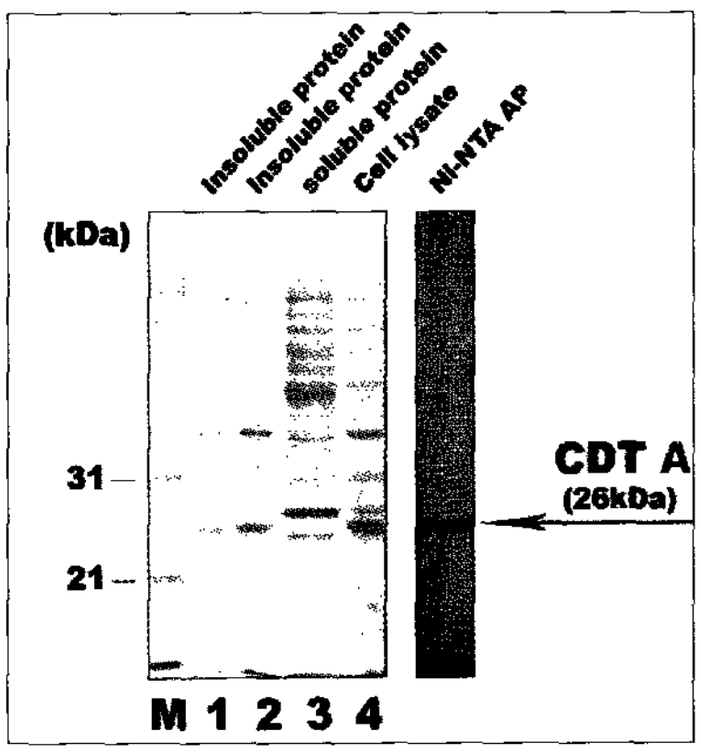

Figure 5. CdtA insoluble protein was confirmed and when testing with AP conjugated Ni-NTA, CdtA protein of 26kDa was observed.

resentative sequence alignment was shown in Figure 3. There was no miss match at all.

4. Subcloning into expression vector

The *cdtA* gene was subcloned into pRSET vector according to the procedures suggested by the manufacturer(Qiagen). And the pRSET -*cdtA* transformed into BL21(DE3) host cells. The cdtA plasmids were isolated and purified for the induction of recombinant protein. Plasmids with expected cdtA gene were prepared from antibiotics-resistant colonies and separated in agarose gel(Figure 4).

5. Expression of recombinant CdtA

After IPTG induction of pRSET-cdt A Expression vector, SDS-PAGE & Western blot proceeded. As a result, CdtA insoluble protein was confirmed and when testing with AP conjugated Ni-NTA, CdtA protein of 26kDa was observed(Figure 5).

IV. DISCUSSION

Actinobacillusactinomycetemcomitans has been implicated not only in the pathogenesis of serious periodontal disease[1], but also in a variety of human systemic diseases such as endocarditis, meningitis and osteomyelitis[2]. Some cytotoxic factors produced by *A. actinomycetemcomitanshave* been studied at the molecular level, and CDT from this pathogen has been identified and cloned[7,8,9].

The CDT action was first described in a culture of eukaryotic cells challenged with supernatants from Campylobacter spp. and enteropathogenic *E. coli*[15,26,27]. Both species caused growth arrest of the intoxicated cells, along with slow distension, over a period of 2 to 5 days. It is now known that CDT is produced by a number of Gram-negative pathogenic bacteria, including Haemophilus ducreyi, *Shigella dysenteriae*, *Helicobacter hepaticus*, as well as *A. actinomycetemcomitans*[10-18]. The growth inhibition and morphological alterations caused by the toxin have been demonstrated in fibroblastic[18,26,27], epithelial or endothelial cells[18,35,36,37,38], as well as T- and B-lymphocytes[19,39,40].

The periodontium is composed of a number of distinct cell types including epithelial cells, fibroblasts, cementoblasts and osteoblasts. From a functional standpoint, the periodontium can be broadly broken down into two tissue compartments referred to as gingiva and the attachment apparatus. Gingiva, which is composed of epithelial cells and the adjacent connective tissue, is attached to a tooth via hemidesmosomes formed by cells of the junctional epithelium. This provides a physical barrier that protects the underlying attachment apparatus from the potentially harmful host as well as microbial and/or environmental factors found within the oral cavity proper. The connective tissues of the periodontium are the gingival connective tissue and the periodontal ligament (PDL) tissue. Gingival fibroblasts (GF) comprise the major cell population of the gingival connective tissue and are respon-

sible for the production of the extracellular matrix of the tissue in health and disease[41]. The PDL is a physically small but very important connective tissue, since it links the alveolar bone to the tooth surface, providing the appropriate tooth support in response to mechanical loading[42]. It consists of a variety of fibroblast cell populations (most commonly designated as PDL cells) with different functional characteristics[43,44]. Both the gingival connective tissue and PDL are likely to be targeted by *A. actinomycetemcomitan*[45,46]. Belibasakis et al. (2002) demonstrated that CDT of *A. actinomycetemcomitans* is involved in the non-lethal inhibition of proliferation of human periodontal ligament fibroblasts and human gingival fibroblasts. Belibasakis et al. (2004) suggested that inhibition was associated with cell-cycle arrest at both the G0/G1 and G2/M phases of growth[47]. However, Kanno et al. (2005) suggested that it is extremely difficult to evaluate these results because: 1) these investigators did not attempt to identify either cdt genes or Cdt polypeptides in their test strain *A. actinomycetemcomitans* HK 1519; 2) their bacterial extract was a crude preparation contaminated with significant levels of LPS (6.5 μ g/ml); 3) the concentration of extract used in their assays was not reported; and 4) effects on cell cycle and evaluation of DNA damage, standard measures of CDT activity, were not determined. Kanno et al.(2005) showed that Human periodontal ligament fibroblasts (HPLF) are resistant to the cytotoxic effects of the *A. actinomycetemcomitans* CDT. Belibasakis et al. (2005) demonstrated that *A. actinomycetemcomitans* induces IL-6 production in GF by a mechanism largely independent of its Cdt and *A. actinomycetemcomitans*-induced RANKL expression in GF occurs independently of IL-1, IL-6, TNF-α, or PGE2.

The crystal structure of the holotoxin from Haemophilus ducreyi reveals that CDT consists of an enzyme of the DNase-I family, bound to two ricin-like lectin domains. The lectin subunits form a deeply grooved, highly aromatic surface. The holotoxin possesses a steric block of the CdtB active site by means of a non-globular extension of the CdtC subunit[50]. *A. actinomycetemcomitans* CdtA has a signal peptidase II recognition site that is normally found on lipoproteins, suggesting that this protein may be anchored to the bacterial outer membrane[7]. CdtA labelled with either a fluorophore[34] or with biotin[33] has been found to localize to selected areas of the plasma membrane of mammalian cells.

V. CONCLUSION

It is not clear what role CdtA plays in the final activity of CDT, and further work is required to fully identify the role of CdtA and its mechanism of host cell binding. The vaccine development to target *A. actinomycetemcomitans cdtA* and the early immunization during the time span of the window of infectivity will have a synergistic effect on suppressing internalization of *A. actinomycetemcomitans* CDT. For this pur-

pose, I cloned *cdtA* from *A. actinomycetemcomitans* (Y4), and subcloned *cdtA*, expressed and purified to homogeneity the CdtA protein as 6×His tag fusion protein. Next, I wll try to investigate the biological activities of CdtA in bacterial pathogenesis, and also to produce the CdtA antibody for vaccine development.

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-국문초록-

*Actinobacillus actinomycetemcomitans*의 cytolethal distending toxin subunit CdtA 유전자 클로닝과 단백질 발현

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Cytolethal distending toxin(CDT)은 세포 주기 중 G2에서 M 기로의 전환을 막아 세포의 증식을 억제할 수 있는 세균 단백 독소의 일종이다. 구강 미생물 중 유일하게 *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*)만이 이 CDT를 생성 할 수 있는 것으로 알려져 있다. *A. actinomycetemcomitans*는 localized aggressive periodontitis (LAP)의 원인균으로 여겨지며 비 운동성의 그람 음성 구간균이고 37℃, 5% CO_2 하에 성장이 왕성하다.

A. actinomycetemcomitans 의 CDT는 3개의 인접한 유전자인 cdtA, cdtB, cdtC 에 의해 형성 되며 각각의 유전자에 대한 단백질의 기능은 아직 완전히 밝혀지지 않았다. 현재까지 연구에 의하면 cdtA는 CDT의 세포부착과 관련이 있는 것으로 여겨지며 이 유전자의 기능 이상 시 CDT 의 독성 효과가 현저히 감소한다고 알려져 있다. 따라서 본 연구는 A. actinomycetemcomitans 의 *cdtA* 유전자를 클로닝, 단백질 발현하여 향후 치주질환의 발병과정에서 CdtA의 역할을 규명하고 질환의 예방 및 치료법에 도움을 주고자 하였다.

A. actinomycetemcomitans Y4 균주를 *cdtA* 유전자 클로닝을 위해 사용하였다. *A. actinomycetemcomitans* 의 genomic DNA는 genomic DNA 추출 kit를 사용하여 분리하고 cdtA에 특이적인 primer를 이용하여 PCR을 통해 *cdtA* 유전자를 증폭하였다. 증폭된 cdtA 유전자를 T-vector에 클로닝 하였으며, 클로닝 된 *cdtA* 유전자는 단백질 발현을 위해 pRSET A vector에 서브클로닝 한 후 발현 균주인 BL21(DE3)를 이용하여 발현시켰다. 발현 후 Ni-NTA AP conjugate를 이용한 Western blot을 통해 pRSET-CDTA를 확인하였다.

Key worlds: *Actinobacillus actinomycetemcomitans*, Cytolethal distending toxin, cloning