

# The Cell Cycle-Specific Growth-Inhibitory Factor Produced by *Actinobacillus actinomycetemcomitans* Is a Cytolethal Distending Toxin

MOTOYUKI SUGAI,<sup>1\*</sup> TORU KAWAMOTO,<sup>1,2</sup> SYLVIE Y. PÉRÈS,<sup>3</sup> YOKO UENO,<sup>1</sup>  
HITOSHI KOMATSUZAWA,<sup>1</sup> TAMAKI FUJIWARA,<sup>1</sup> HIDEMI KURIHARA,<sup>2</sup>  
HIDEKAZU SUGINAKA,<sup>1</sup> AND ERIC OSWALD<sup>3</sup>

Department of Microbiology<sup>1</sup> and Department of Periodontology and Endodontology,<sup>2</sup> Hiroshima University School of Dentistry, Hiroshima 734-8553, Japan, and Unité Associée INRA de Microbiologie Moléculaire, Ecole Nationale Vétérinaire de Toulouse, 31076 Toulouse Cedex, France<sup>3</sup>

Received 8 April 1998/Returned for modification 3 June 1998/Accepted 10 July 1998

*Actinobacillus actinomycetemcomitans* has been shown to produce a soluble cytotoxic factor(s) distinct from leukotoxin. We have identified in *A. actinomycetemcomitans* Y4 a cluster of genes encoding a cytolethal distending toxin (CDT). This new member of the CDT family is similar to the CDT produced by *Haemophilus ducreyi*. The CDT from *A. actinomycetemcomitans* was produced in *Escherichia coli* and was able to induce cell distension, growth arrest in G<sub>2</sub>/M phase, nucleus swelling, and chromatin fragmentation in HeLa cells. The three proteins, CDTA, -B and -C, encoded by the *cdt* locus were all required for toxin activity. Antiserum raised against recombinant CDTC completely inhibited the cytotoxic activity of culture supernatant and cell homogenate fractions of *A. actinomycetemcomitans* Y4. These results strongly suggest that the CDT is responsible for the cytotoxic activity present in the culture supernatant and cell homogenate fractions of *A. actinomycetemcomitans* Y4. This CDT is a new putative virulence factor of *A. actinomycetemcomitans* and may play a role in the pathogenesis of periodontal diseases.

*Actinobacillus actinomycetemcomitans* is a gram-negative bacterium belonging to the family Pasteurellaceae. It has been implicated in the pathogenesis of juvenile and adult periodontitis (44, 45, 55). *A. actinomycetemcomitans* cells adhere to human cells, and some of them eventually invade the attached cells in vitro (8, 25). They produce a variety of virulence factors, including cytotoxic factors (1, 9, 10, 15–17, 24, 41–43, 46, 50, 52), chemotactic inhibitor (49), collagenases (36), and lipopolysaccharide (19, 37), but little is known about the roles of such virulence factors in the pathogenesis of periodontal diseases at the molecular level. Among cytotoxic factors, leukotoxin has been most extensively studied (20–23, 26, 55), but there are several reports concerning the existence of other cytotoxic factors produced by *A. actinomycetemcomitans* (10, 15, 41–43). Although the possible involvement of these factors in the pathogenesis of periodontitis has been suggested, their exact role has yet to be elucidated.

We have now identified a cluster of genes in *A. actinomycetemcomitans* that encode proteins belonging to the family of the cytolethal distending toxin (CDT), which is produced by certain pathogenic *Escherichia coli* strains (29, 31, 40), *Campylobacter* species (32), *Shigella* species (28), and *Haemophilus ducreyi* (7). CDT has recently been shown to block the cell cycle of HeLa cells (29). In this report, we demonstrate that *A. actinomycetemcomitans* produces a new member of the CDT family which also induced growth arrest in G<sub>2</sub>/M phase of cultured HeLa cells.

## MATERIALS AND METHODS

**Materials and chemicals.** All restriction enzymes, T4 DNA ligase, and Klenow fragment of DNA polymerase I were from Boehringer Mannheim, Tokyo, Japan,

or New England BioLabs, Inc., Beverly, Mass. Other materials and chemicals used were from commercial sources.

**Bacterial strains and culture conditions.** *A. actinomycetemcomitans* Y4 (serotype b, ATCC 43718) was cultured in Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 1% (wt/vol) yeast extract in a 5% CO<sub>2</sub> atmosphere. *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium or on LB agar plates. Ampicillin (50 µg/ml) or kanamycin (50 µg/ml) was used when appropriate. Manipulation of DNA in *E. coli* was carried out with pUC19 (54), pGEM-T Easy (Promega, Madison, Wis.), or pET-28a(+) (Novagen, Madison, Wis.).

**Cells and culture conditions.** HeLa cells (ATCC CCL2) were grown in Eagle's minimal essential medium (Nissui) supplemented with 10% fetal bovine serum at 37°C and in a 5% CO<sub>2</sub>-95% air atmosphere.

**Detection of cytodistending activity in bacterial sterile lysates.** Bacterial cells were recovered from cultures of *A. actinomycetemcomitans* strains or *E. coli* recombinant strains by centrifugation (10,000 × g, 20 min), and the pellets were resuspended in phosphate-buffered saline (PBS) to an optical density at 660 nm of 0.1. A cell suspension of 1 ml was lysed by periodic sonication for 30 s six times in ice (Ultradruptor; TOMY SEIKO, Tokyo, Japan). After clarification by centrifugation (10,000 × g, 20 min), lysates or culture supernatants were filtered (0.2-µm-pore-size filter) and placed on HeLa cell monolayers in a 48-well plate (Falcon; Becton Dickinson) (1.6 × 10<sup>3</sup> cells per well). The occurrence of cytotoxic effects was monitored up to day 5. Cytodistending activity was titrated by using as the end point the highest twofold dilution of toxic material giving 50% transformed cells after 72 h of incubation (50% cytotoxic dose [CD<sub>50</sub>]).

**DNA manipulations.** Routine DNA manipulations, DNA digestion with restriction enzymes, DNA ligations, gel electrophoresis, Southern blotting of DNA and hybridization, and DNA sequencing were performed essentially as described previously (38). Purification of chromosomal DNA from *A. actinomycetemcomitans* was performed as described previously (38). Restricted genomic DNA was separated by electrophoresis overnight in a 1% agarose gel. DNA fragments of 5 to 10 kb were recovered by electroelution and ligated to *Eco*RI-digested and alkaline phosphatase-treated pUC19. The ligated DNA was transformed into *E. coli* XL-1 Blue, and the transformants were selected on LB agar that contained ampicillin (50 µg/ml). Hybridization was performed by means of an enhanced chemiluminescence procedure (ECL direct labelling kit or 3'-oligolabelling kit; Amersham Life Science, Buckinghamshire, United Kingdom). DNA sequences of both strands were determined by the dideoxy-chain termination method (39) with an Auto-Read sequencing kit (Pharmacia Biotech, Tokyo, Japan). A nested set of deletions for sequencing was constructed by using exonuclease III and mung bean nuclease (Kilosequence deletion kit; Takara Bio-medicals, Tokyo, Japan) according to the method of Henikoff (11). Extraction of large plasmids from lysed bacteria was carried out by the method of Kado and Liu (14).

\* Corresponding author. Mailing address: Department of Microbiology, Hiroshima University School of Dentistry, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan. Phone: 81 82 257 5637. Fax: 81 82 257 5639. E-mail: sugai@ipc.hiroshima-u.ac.jp/.

TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
XL1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 Lac<sup>-</sup> (F' [proAB<sup>+</sup> lacI<sup>s</sup> lacZ ΔM15 Tn10 {Tet<sup>r</sup>})</i>	5
HMS174	<i>recA1 hsdR Rif<sup>r</sup></i>	47
Plasmids		
pUC19	Cloning vector, Amp <sup>r</sup>	54
pGEM-T Easy	Cloning vector, Amp <sup>r</sup>	Promega
pET-28a(+)	Cloning vector, Kan <sup>r</sup>	Novagen
pTK3003	5.6-kb <i>EcoRI</i> fragment in pUC19	This study
pTK3005	Reverse insertion of 5.6-kb <i>EcoRI</i> fragment in pUC19	This study
pTK3022	3.4-kb <i>SmaI-EcoRI</i> fragment in pUC19	This study
pTK3034	3.7-kb 3' deletion fragment of pTK3003 <i>EcoRI</i> insert	This study
pTK3035	3.65-kb 3' deletion fragment of pTK3003 <i>EcoRI</i> insert	This study
pTK3037	2.9-kb 3' deletion fragment of pTK3003 <i>EcoRI</i> insert	This study
pTK3043	1.9-kb 3' deletion fragment of pTK3003 <i>EcoRI</i> insert	This study
pTK3047	1-kb 3' deletion fragment of pTK3003 <i>EcoRI</i> insert	This study
pTK3046	5.1-kb 3' deletion fragment of pTK3005 <i>EcoRI</i> insert	This study
pTK3045	4.2-kb 3' deletion fragment of pTK3005 <i>EcoRI</i> insert	This study
pTK3251	<i>cdtA</i> (1,093 bases) PCR fragment in pGEM-T Easy	This study
pTK3252	<i>cdtB</i> (1,211 bases) PCR fragment in pGEM-T Easy	This study
pTK3253	<i>cdtC</i> (969 bases) PCR fragment in pGEM-T Easy	This study
pTK3286	<i>cdtA</i> (636 bases) PCR fragment in pET-28a(+)	This study
pTK3287	<i>cdtB</i> (797 bases) PCR fragment in pET-28a(+)	This study
pTK3288	<i>cdtC</i> (561 bases) PCR fragment in pET-28a(+)	This study

**PCR.** PCR reagents were from Perkin-Elmer (Norwalk, Conn.), and PCR was performed with the GeneAmp PCR System 2400 (Perkin-Elmer). Primers were supplied by Greiner Japan Co. (Tokyo, Japan). The primer sets used are listed in Table 2.

**Purification of recombinant His<sub>6</sub>-tagged protein.** *E. coli* HMS174 (47) carrying a plasmid was grown at 37°C with vigorous shaking until an optical density of 0.5 was reached, and then the expression of His<sub>6</sub> protein was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 5 h of incubation, bacteria were precipitated by centrifugation, resuspended in 20 mM Tris-HCl containing 0.5 M NaCl and 50 mM imidazole (pH 7.9) (buffer 1), and disrupted with an Ultrasonic disruptor (TOMY SEIKO). After centrifugation at 9,000 × g for 30 min, the pellet was treated with buffer 1 containing 8 M urea. The recombinant His<sub>6</sub>-tagged protein was purified by Ni-chelated affinity chromatography. A TSKgel AF-chelate 5PW column (7.5 by 75 mm) was pretreated with 6 column volumes of 50 mM NiSO<sub>4</sub> and equilibrated with buffer 1 containing 8 M urea. After removal of the debris from the sonicated sample by centrifugation at 37,500 × g for 30 min, the supernatant was applied to the column until most of the unbound proteins passed through. Bound proteins were eluted with a linear gradient from buffer 1 to 20 mM Tris-HCl buffer containing 0.5 M NaCl and 1 M imidazole (pH 7.9) (buffer 2) at a flow rate of 1 ml/min in 30 min. The protein samples obtained were subjected to disc preparative electrophoresis (NA-1800; Nihon Eido, Tokyo, Japan), and fractions showing a single protein band by analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used for immunization with rabbits.

**Antiserum.** The purified recombinant His<sub>6</sub>-tagged proteins were emulsified with either Freund complete or incomplete adjuvant (Difco Laboratories, Detroit, Mich.) (100 μg of protein per ml). For each sample, 2-kg rabbits were immunized at day 1 with sample emulsified with Freund complete adjuvant and at 2 weeks with sample emulsified with Freund incomplete adjuvant. At 4 weeks, the rabbits were injected intravenously with 50 μg of protein from the sample. Antisera were obtained 5 weeks after first injection. Preimmune serum was used as control serum.

**In vitro transcription and translation.** In vitro synthesis of proteins from plasmid DNA templates was performed by using the *E. coli* S30 Extract system (Promega) with EXPRE<sup>35</sup>S<sup>35</sup>S protein labelling mix (1,000 Ci/mmol; DuPont-

NEN Research Products, Boston, Mass.) according to the manufacturer's protocol.

**Demonstration of HeLa cell morphological and nuclear changes.** At intervals, HeLa cells were monitored by phase-contrast microscopy with photographs taken to document observations. Also, morphological and nuclear changes were demonstrated by means of staining with Giemsa stain or propidium iodide (PI) and Hoechst 33342 as described previously (29).

**Flow cytometry analysis.** After trypsinization and washing in PBS (pH 7.2), cells were fixed in 1% formaldehyde in PBS for 15 min on ice. Then, after three washes in PBS, cells were suspended in 70% ice-cold ethanol and immediately transferred to -20°C until ready for use. After fixation, the cells were rehydrated in PBS at room temperature, permeabilized with Triton X-100, and then incubated in the dark at 4°C for 30 min in 1 ml of a PBS solution containing RNase (1 mg/ml) and PI (10 μg/ml). Flow cytometric analysis of the DNA content was performed on a FACScalibur flow cytometer (Becton Dickinson). The data from 2 × 10<sup>4</sup> cells were collected and analyzed with CellQuest software. In this experiment, flow cytometer parameters were adjusted to obtain G<sub>1</sub> (2n DNA content) and G<sub>2</sub> (4n DNA content) cell cycle peaks of control cells, centered at 200 and 400 DNA units (DU), respectively. Flow cytometric analysis of the DNA contents from different samples was repeated three times in independent tests.

**Other procedures.** SDS-PAGE and Western blotting (immunoblotting) were carried out as described previously (48). Protein was immunodetected by using Renaissance 4CN plus (Dupont-NEN). Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.), with bovine serum albumin as the standard.

**Nucleotide sequence accession number.** The nucleotide sequence data presented in this report will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB011405.

## RESULTS

***A. actinomycetemcomitans* Y4 induces distension and cell cycle blockage in G<sub>2</sub>/M phase in HeLa cells.** HeLa cells were treated with a sterile sonic lysate of *A. actinomycetemcomitans* Y4 as described in Materials and Methods. After 3 days of incubation, a cytopathic effect with distension in cell size was observed in treated cells (Fig. 1A, panel b). The mean size of distended cells was four- to fivefold that of control cells. The size of cell nuclei started to increase after 1 day of incubation. The mean diameter of nuclei was 1.4-fold that of control cells. When the incubation time was extended, cells started to detach from the culture dish. Flow cytometry analysis of the DNA content of HeLa cells revealed that the number of cells in G<sub>1</sub> decreased while the number of cells in G<sub>2</sub>/M increased (Fig. 1B). These results suggested that the cells treated with the lysate of *A. actinomycetemcomitans* Y4 were blocked in the G<sub>2</sub>/M phase of the cell cycle. After 3 days of incubation, some populations of cells revealed fragmented nuclei with condensed masses of chromatin (Fig. 1A, panel b), which was often observed with apoptotic cells. Similar results were obtained with culture supernatant of *A. actinomycetemcomitans* Y4 (not shown). The cytodistension titer of the culture super-

TABLE 2. Primers used in this study

Primer	Sequence <sup>a</sup>	Position
MIX5'	5'-GAAARYAAATGGARYRYWMTGTMMG-3'	
MIX3'	5'-AAATCWCWRSAAATCAATCCAGTTA-3'	
AASPC5'	5'-AAAGTAAATGGAATATTAATGTGCG-3'	1475-1499
AASPC3'	5'-AAATCACCACCAACCATCCAGCTA-3'	1926-1949
U257	5'-TGCTGAACAAGTATACATTCGTAAAGAA-3'	257-284
L1323	5'-ATAAACTCCTTAGCTTAATTAACCGCTG-3'	1323-1350
U1001	5'-ATTCGTAATTGGAAGATAGAACCCTGG-3'	1001-1026
L2188	5'-AGTATTCTCCTTAGCGATCATGAAC-3'	2188-2212
U1851	5'-TGATGCGGTAAGTTAATTCGTAATA-3'	1851-1875
L2801	5'-CCCTCGCCCCACTAAGCATCTTGATT-3'	2801-2826
U714	5'-GTTTCGTCAAATCAACGAATGAGTGACT-3'	714-740
U1415	5'-CTAACTTGAGTGATTTCAAAGTAGCA-3'	1415-1440
U2265	5'-GTCATGCAGAATCAAATCCTGATCCG-3'	2265-2290

<sup>a</sup> R is A or G, Y is C or T, M is A or C, W is A or T, and S is G or C.

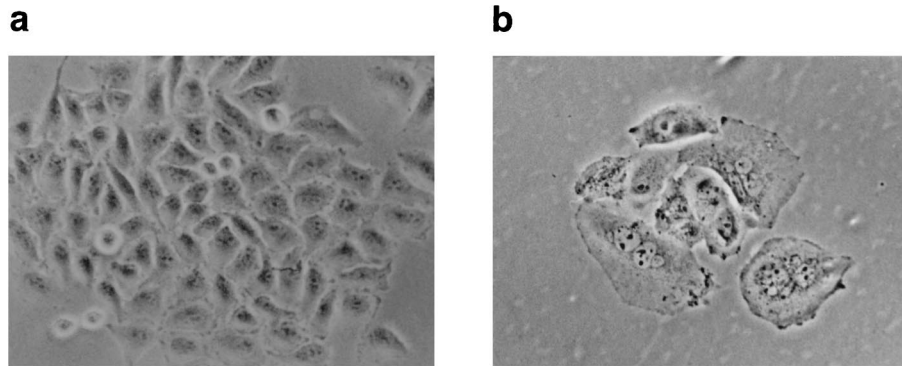
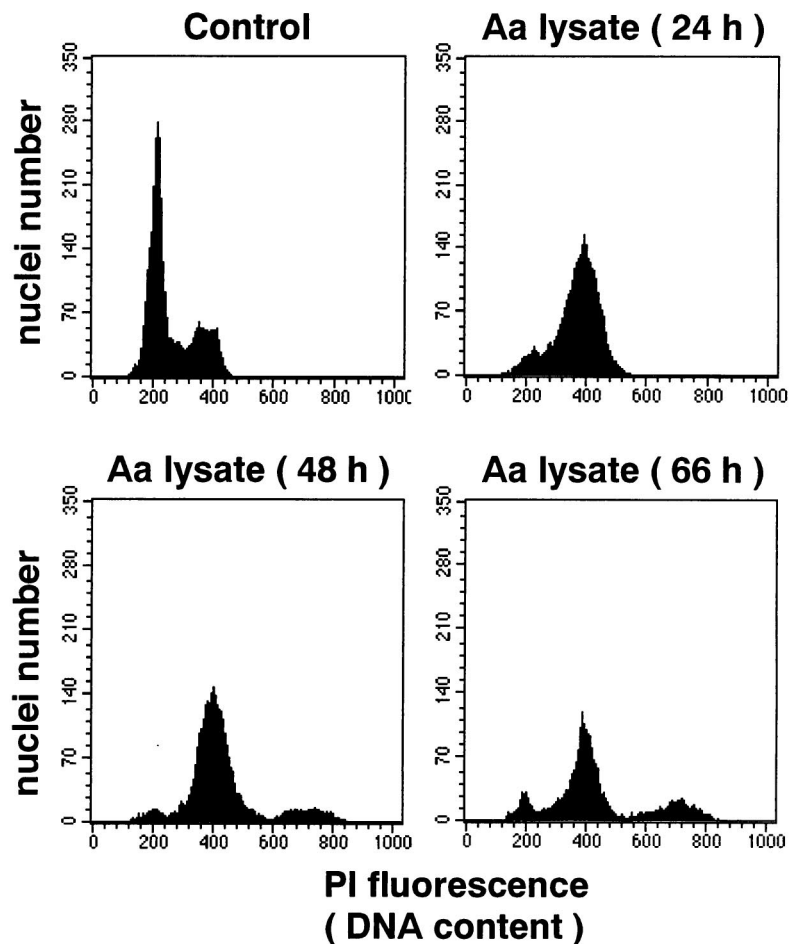
**A****B**

FIG. 1. Effect of *A. actinomycetemcomitans* Y4 sonic lysate on cultured HeLa cells. (A) HeLa cells 3 days after incubation with (b) or without (a) 4 CD<sub>50</sub> of sterile sonic lysate of *A. actinomycetemcomitans* Y4. Magnification,  $\times 150$ . (B) Cell cycle pattern of HeLa cells incubated with 4 CD<sub>50</sub> of sterile sonic lysate of *A. actinomycetemcomitans* Y4 (Aa lysate) for the indicated times. Control, cells without treatment.

nant was similar to that of the sonic lysate (18 versus 32 CD<sub>50</sub> per mg of protein).

**Cloning and genetic analysis of the *A. actinomycetemcomitans* Y4 *cdt* genes.** From these observations, we hypothesized that *A. actinomycetemcomitans* Y4 produces a CDT. Degener-

ative oligonucleotide primers, MIX5' and MIX3', were designed according to the homology observed between the different *cdtB* genes cloned so far. These primers were then tested in PCR experiments with *A. actinomycetemcomitans* Y4 template DNA. A single product of approximately 475 bp was amplified.

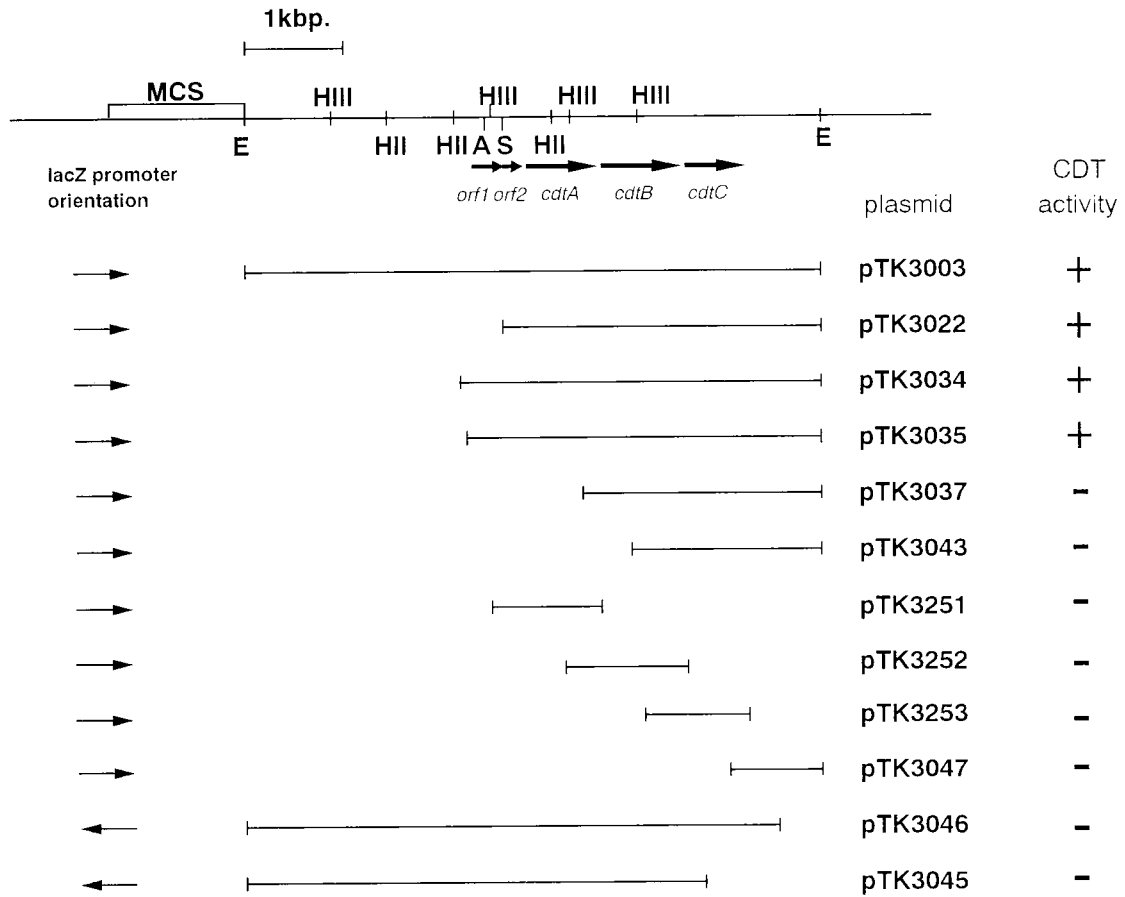


FIG. 2. Restriction map of subclones from *A. actinomycetemcomitans* DNA that contains *cdtABC*. Arrows represent ORFs and directions of transcription. CDT activity was measured by incubating sterile sonic lysates of recombinant strains with HeLa cell cultures for 3 days. Clones expressing CDT activity are indicated at the right. The restriction sites of relevant endonucleases are indicated: HIII, *HindIII*; HII, *HincII*; E, *EcoRI*; A, *AccI*; S, *SmaI*. MCS, multicloning site for cloning vector.

Since this was close to the expected size of 460 bp, the product was cloned into pGEM-T Easy and sequenced. The nucleotide sequence obtained indicated that the relevant portion of the *cdtB* gene had been amplified. Specific primers, AASPC5' and AASPC3', then were generated, and the resulting PCR product was used as a DNA probe to identify one clone containing *cdt* genes in our *A. actinomycetemcomitans* Y4 DNA library. This clone carried a recombinant plasmid with a 5.6-kb *EcoRI* DNA fragment in pUC19. This plasmid was called pTK3003, and a lysate of the pTK3003 clone was found to produce CDT. A restriction map of the 5.6-kb *EcoRI* fragment was established (Fig. 2). To determine the minimum amount of DNA required for the CDT activity, a deletion series of the *EcoRI* fragment was constructed. An assay of deletion clones for cytodistending activity revealed that bacteria carrying pTK3022, pTK3034, and pTK3035 expressed cytodistending activity. The other clones listed in Fig. 2 did not show any cytodistending activity. Fragments expressing cytodistending activity were sequenced by using either the universal or the reverse sequencing primer. The locations of the open reading frames (ORFs) are shown in Fig. 2, and the nucleotide sequence for 3,560 bp of DNA, including the entire ORFs, together with the deduced amino acid sequence is shown in Fig. 3.

The *cdt* genes are arranged in a manner similar to those of other species (7, 28, 29, 31, 32, 40). There are three adjacent ORFs which encode putative CDTA, CDTB, and CDTC proteins. The *cdtA* and *cdtB* genes were separated by 17 nucleotides,

and the *cdtB* and *cdtC* genes were separated by 13 nucleotides. The percent G+C contents of the *A. actinomycetemcomitans* *cdtA*, *cdtB*, and *cdtC* genes were 38.4, 41.3, and 37.8% respectively, which is similar to the G+C content determined for *A. actinomycetemcomitans* chromosomal DNA (42.7%) (30). Three putative Shine-Dalgarno sequences were located upstream of the coding sequences.

The deduced molecular masses of the proteins encoded by these three ORFs are 24,512, 31,492 and 20,706 Da, respectively. A possible signal peptide was present in each protein, and each of these had a hydrophobic region of 10 to 12 amino acids at the N terminus that was followed by 1 or 2 basic amino acids. The possible signal sequence for the first ORF revealed the presence of a consensus sequence, LVAC, for prolipoprotein modification and processing by signal peptidase II (4, 53). On the other hand, those for the second and third ORFs were terminated by a sequence resembling that for processing by signal peptidase I. Comparison of the predicted amino acid sequences with known sequences in the DDBJ database revealed that these ORFs code for a new member of the CDT family and are very similar to those of *H. ducreyi* CDTs (7) (Table 3).

Besides the *cdt* locus, we found two putative ORFs, ORF1 and ORF2, upstream of *cdtA*. Interestingly, ORF1 shows significant homology at the amino acid level with the hypothetical protein HI0321 of *Haemophilus influenzae* (GenBank accession no. U32717) and with the VagC, VapB, and STBORF1

GTCAACGAAGCTCCCAAGAAGCTCAAATTTACCTCATCGACCCAAACCCGAACACAGGGTTAATCCTGTTACTGGGTGGTGAACGTCTTTTGCGATAAA 100  
 CAGATAAGTTTTGACTCTTTTATGGTTATAGCATACGTTTGGCATATATATCAATCTTGGACTTAAAATACAGGAGGAGACATGGAAGAAGTGGCGAAA 200  
 TTATTCAGAAACGGACGCAATCAAGCCGTACGGTTACCGGTGGAGTTCGAATTTGATGCTGAACAAGTATACATTCGTAAGAACAATAATGGAGATATTG 300  
 L F R N G R N Q A V R L P V E F E F D A E Q V Y I R K E Q N G D I V  
 TACTTTCATTGACTTCTTCAAGAGAAGCTTCTTGGCAGCGGTTGTTTAAGTTACTTCCCAAAATAAAAAATGTGATGATTTTCTCAGCAAAGAAGAGCG 400  
 L S L T S S R E A S W Q R L F K L L P K I K N C D D F L S K E E R  
 GAATCAAACGATTACGACCCGTTACCGGTTCCCGGGATAGGTGAATAATAGATGTATATGCTTGATACTAACACGGTAAGTTATATTTCTACTTAACA 500  
 N Q T I T T R Y P F P G I G E M Y M L D T N T V S Y I S Y L T  
 GATTTATTCATATTTTATTTCACAGATTTCAAAAATATATGAATCAAAAACAAATTCCTCTTCTTTTATATATTTTATCTAGTTTATTTTAT 600  
 D L F I F L F Q Q I S K T I L N Q K T I P L P F L Y I L S S F I L S  
 CAAAAAAGGATAAAAATAAGAAAAATTTGCCCTTTTAAATTAAGGATGGATCTAAGGAGGTTTACAATGAAAAAGTTTACTGGTCTTTTATGATG 700  
 K K G SD H K K F L P G L L L H  
 GGTTAGTGGCTTGTTCGTCAAATCAACGAATGAGTGACTATCTCAGCCTGAATCTCAATCTGATTTAGCACCTAAATCTTCAACAACACAATTCACAC 800  
 G L V A C S S N Q R M S D Y S Q P E S Q S D L A P K S S T T Q F Q P  
 CCAACCCCTATTATCAAAAGCATCTTCAATGCCATTGAATTTGCTCTCTTCAATCAAGAATGGACAGGTATCGCCGTCTGAACCATCAAACTTTATGAC 900  
 Q P L L S K A S S M P L N L L S S S K N G Q V S P S E P S N F M T  
 TTTGATGGGACAAAATGGGCACTGTTGACTGTCTGGGCGCTAGCAAAACGCAATGGTTATGGGCTTATCCCAATATATATTCGCAGGACTTTGGAAT 1000  
 L H G Q N G A L L T V W A L A K R N W L W A Y P N I Y S Q D F G N  
U1001  
 ATTCGTAATTGGAAGATAGAACCTGGTAAACCCGTAATATTTTCGTTTTGTTAATCAATCTTTAGGTACATGTATTGAAGCTTACGGTAATGGTTAA 1100  
 I R N W K I E P G K H R E Y F R F V N Q S L G T C I E A Y G N G L I  
 TTCATGATACTTGTAGTCTGGACAATAAGTAGCACAAGAGTTTGTAGTTATTACCTACTGATAGTGGTGGTGTGTCATTAAGAGTGTGCACAAGGACGTTG 1200  
 H D T C S L D K L A Q E F E L L P T D S G A V V I K S V S Q G R C  
 TGCTACTTATAATCCTGTAAGTCCAACATATTATCAACAGTTACATTATCAACTTGTGATGGCGCAACAGAACCATACGTTGATCAAAATGGTATCTC 1300  
 V T Y N P V S P T Y Y S T V T L S T C D G A T E P L R D Q T W Y L  
 GCTCCTCTGATTAGAAGCAACAGCGTTAATTAAGCTAAGGAGTTTATATGCAATGGGTAAAGCAATTAATGTGGTTTTCTGTACGATGTTATTTAG 1400  
 A P P V L E A T A V N SD M Q W V K Q L N V V F C T H L F S  
 CTTTTCAAGTTATGCTAACTTGAGTGATTTCAAAGTAGCAACTTGGAACTGCAAGGTTCTTCAAGCTGAAATGAAAGTAAATGGAATATTAATGTGGCG 1500  
 F S S Y A N L S D F K V A T W N L Q G S S A V N E S K W N I N V R  
 CAATTATTATCGGGAGAACAAGGTGCAGATATTTTGTGTTGTTACAAGAAGCGGGTTCATTACCAAGTTCGGCAGTAAGAACCCTCAGGTAATTCACATG 1600  
 Q L L S G E Q G A D I L M V Q E A G S L P S S A V R T S R V I Q H G  
 GGGGAACGCCAATTGAGGAATATACCTGGAATTTAGTACTCGCTCCCGTCCAATATGGTCTATATTTATTATTTCCCGTTAGATGTTGGGGCAAACCG 1700  
 G T P I E E Y T W N L G T R S R P N M V Y I Y Y S R L D V G A N R  
 AGTGAACCTAGCTATCGTGTACGTCAGCTGCAAGCCGATGAAGCTTTTATCGTACATCTGATTCCTCTGTGCTTCAATCTCGCCCGCAGTAGGTATCCGC 1800  
 V N L A I V S R R Q A D E A F I V H S D S S V L Q S R P A V G I R  
U1851  
 ATGGTACTGATGATTTTTTACAGTGCATGCTTTGGCCACAGGTGGTTCTGATGCGGTAAGTTTAATTCGTAATATCTTCACTACTTTTACCTCATCAC 1900  
 I G T D V F F T V H A L A T G G S D A V S L I R N I F T T F T S S P  
 CATCATCACCGAAAGACGAGGATATAGCTGGATGTTGTTGGTGAATTTCAATCGTCCGCGGTTAATCTGGAAGCTGCATTAAGACAGGAACCCGCCGT 2000  
 S S P E R R G Y S W M V V G D F N R A P V N L E A A L R Q E P A V  
 GAGTGAATAACAATTATTATTTGCGCAACAGAACCGACTCATCGTCCGGTAATATTTTAGATTATGCGATTTTACATGACGCACATTTACCACGTCGA 2100  
 S E N T I I I A P T E P T H R S G N I L D Y A I L H D A H L P R R  
 GAGCAAGCACGTGAACGTATCGGGCAAGTTTAAATGTTAAATCAGTTACGCTCACAAATTACATCCGATCATTTTCTGTTAGTTTTGTTTCATGATCGCT 2200  
 E Q A R E R I G A S L M L N Q L R S Q I T S D H F P V S F V H D R

FIG. 3. DNA sequence analysis in the region of the *HincII-EcoRI* site of the cloned DNA fragment. ORF1, ORF2, and three complete ORFs defining *cdtA*, *-B*, and *-C* were found. Putative ribosome binding (Shine-Dalgarno [SD]) sites for *cdt* are boxed. Putative signal peptide cleavage sites are indicated (▲). Oligonucleotide primers used are labelled and are indicated by arrows above the relevant sequences. A DNA sequence similar to that of the integrating plasmid of *H. influenzae* is underlined.



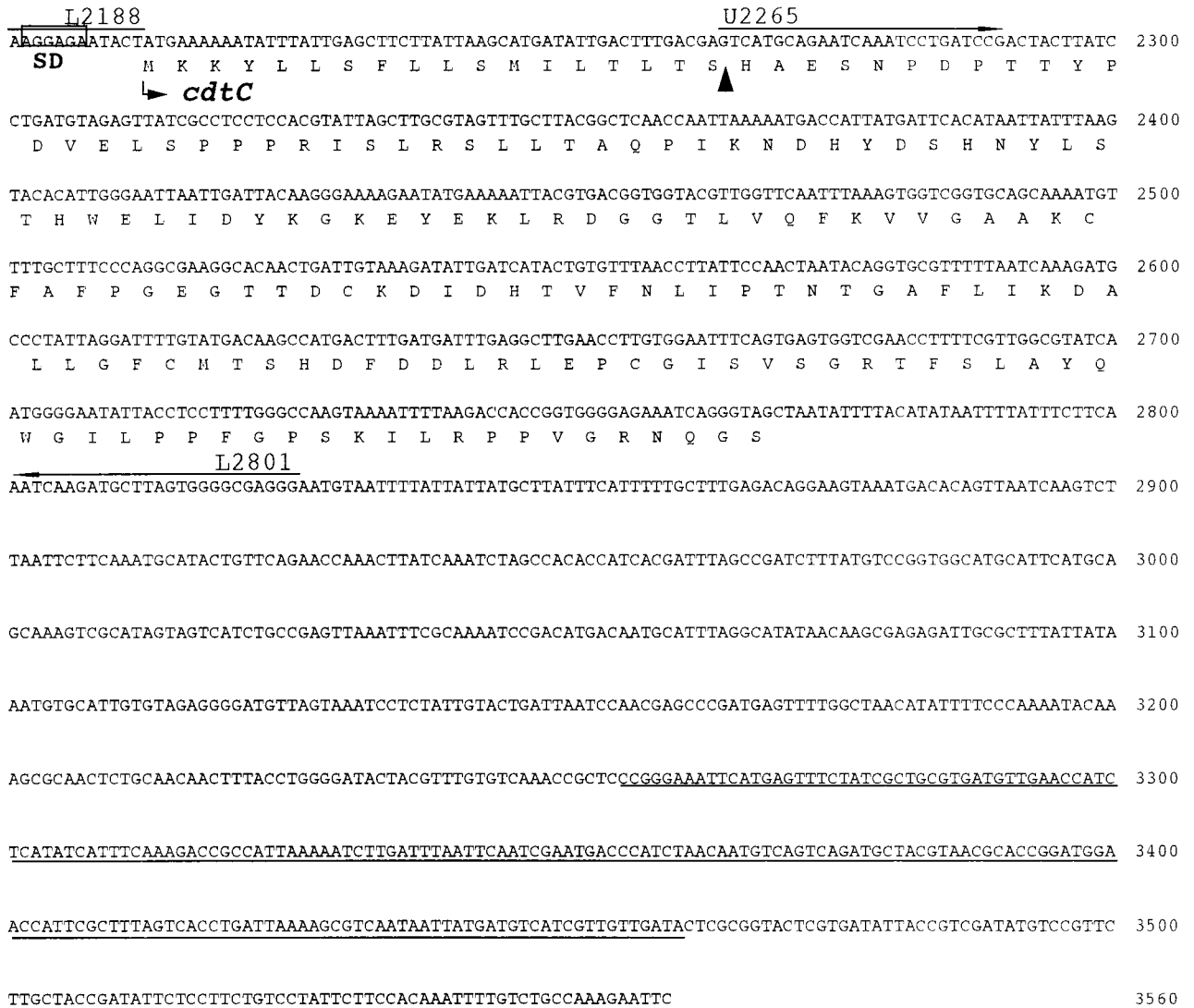


FIG. 3—Continued.

proteins, which are involved, respectively, in the stability of virulence plasmids in *Dichelobacter nodosus* (2), *Salmonella dublin* (33), and *Shigella flexneri* (35). In addition, we also found downstream of the *cdt* locus a small DNA stretch homologous to an integrating plasmid of *H. influenzae* (GenBank accession no. U68467). Taken together, these results raised the possibility that the *cdt* genes of *A. actinomycetemcomitans* were located on a plasmid, like the *cdtIII* genes carried by the Vir plasmid of *E. coli* (29). Plasmid DNA was therefore extracted from lysed *A. actinomycetemcomitans* Y4 by the method described by Kado and Liu (14). However, there was no visible band observed in agarose gel electrophoresis. We further tested whether the *cdt* genes are encoded on a plasmid by Southern hybridization. The PCR product obtained with specific primers, AASPC5' and AASPC3', was used as a probe. Southern hybridization of the extract prepared by the Kado-Liu method (14) failed to show any signal, suggesting that the *cdt* genes were not located on a plasmid (not shown).

**Expression and toxic properties of *A. actinomycetemcomitans* Y4 *cdt* gene products.** A clone containing all three *cdt* genes was constructed by inserting a 3.4-kb *SmaI-EcoRI* fragment into pUC19 (54). The resulting clone, pTK3022, contained in-

tact *cdtA*, *cdtB*, and *cdtC* and produced CDT activity in *E. coli* XL-1 Blue (5) (Fig. 2). To confirm the presence of the three ORFs and identify protein products for each, we performed in vitro transcription-translation studies with DNA fragments

TABLE 3. Comparison of the predicted amino acid sequences of the three genes of *A. actinomycetemcomitans* CDT and those of CDT loci previously cloned and sequenced

<i>A. actinomycetemcomitans</i> gene product	% Identity with <sup>a</sup> :				
	CDT-I	CDT-II	CDT-III	CDT-c	CDT-h
CDTA	29	22	22	27	91
CDTB	48	45	46	48	97
CDTC	19	23	19	20	94

<sup>a</sup> CDT-I, locus cloned by Scott and Kaper (40) from *E. coli* E6468/62 (GenBank accession no. U03293); CDT-II, locus cloned by Pickett et al. (31) from *E. coli* 9142-88 (GenBank accession no. U04208); CDT-III, locus cloned by Pères et al. (29) from *E. coli* 1404 (GenBank accession no. U89305); CDT-c, locus cloned by Pickett et al. (32) from *C. jejuni* 81-176 (GenBank accession no. U51121); CDT-h, locus cloned by Cope et al. (7) from *H. ducreyi* 35000 (GenBank accession no. U53215).

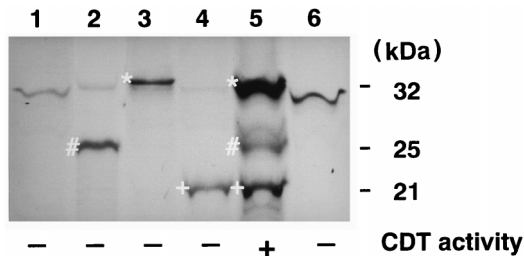


FIG. 4. Autoradiograph of an SDS-12% polyacrylamide gel containing [<sup>35</sup>S] methionine-labelled products of in vitro transcription-translation. Lanes: 1, pGEM-T Easy; 2, pTK3251, containing the *A. actinomycetemcomitans* *cdtA* gene; 3, pTK3252, containing the *A. actinomycetemcomitans* *cdtB* gene; 4, pTK3253, containing the *A. actinomycetemcomitans* *cdtC* gene; 5, pTK3022, containing the entire *cdtABC* gene cluster; 6, pUC19. The CDT activities of sterile sonic lysates from the recombinant strains are indicated at the bottom. Radiolabelled bands marked by #, \*, and + are putative gene products of *cdtA*, *-B*, and *-C*, respectively.

cloned into a plasmid. Each of the *cdt* genes, containing approximately 250 bp of 5' flanking DNA from the start codon, was amplified by PCR with primer sets U257 and L1326, U1001 and L2188, and U1851 and L2801, respectively (Table 2), and cloned into pGEM-T Easy. Proteins with apparent molecular masses of 25, 32, and 21 kDa were expressed by *cdtA*, *cdtB*, and *cdtC*, respectively (Fig. 4). These three protein products were present in pTK3022, which includes all three *cdt* genes, and the estimated molecular masses of the proteins were in good agreement with predicted values. When culture supernatants from these recombinant *E. coli* strains were tested for CDT activity, only that from the strain carrying pTK3022 had CDT activity against HeLa cells (Fig. 2).

The effect of *A. actinomycetemcomitans* CDT on cell morphology and on the DNA content of HeLa cells was studied with a sterile sonic lysate of the pTK3022 clone. The HeLa cells were exposed to a dose equivalent to 20 CD<sub>50</sub>. After 3 days of incubation, treated cells had a distended morphology (Fig. 5A, panel b). The mean size of distended cells was 10- to 18-fold that of control cells. Most of the cells were mononucleated, but a few were binucleated. When the incubation time was extended, most cells started to detach from the culture dish. The DNA content of the HeLa cells treated with *A. actinomycetemcomitans* CDT was analyzed after staining of nuclei with PI and Hoechst 33342, by fluorescence microscopy, and with PI by flow cytometry (Fig. 5B and 6). PI and Hoechst 33342 staining revealed that the nuclei of *A. actinomycetemcomitans* CDT-treated cells increased in size after 3 days of incubation (Fig. 5B, panel b). After 4 days, cells with fragmented nuclei were increased in number (Fig. 5B, panel c). Flow cytometry demonstrated that *A. actinomycetemcomitans* CDT was able to block the cell cycle (Fig. 6). After 24 h, the number of cells in G<sub>2</sub> increased, whereas that of cells in G<sub>1</sub> decreased. At 48 h, the G<sub>2</sub>/M peak slightly decreased but was still dominant. A third DNA peak, centered at 700 DU, appeared at 48 h and slightly increased after 72 h. This third peak theoretically suggests the presence of cells with multiple nuclei (nuclei in G<sub>1</sub> and/or in G<sub>2</sub>). Since PI staining suggested the presence of binucleated cells, the third peak in *A. actinomycetemcomitans*-treated cells is composed mainly of binucleated cells in G<sub>2</sub>/M and, to a lesser extent, those with four nuclei in G<sub>1</sub>. Thus, cells exposed to *A. actinomycetemcomitans* CDT were blocked at the G<sub>2</sub>/M stage.

**Anti-HisCDTC serum inhibits induction of cell distension by *A. actinomycetemcomitans* Y4 cell lysate.** We constructed DNA fragments corresponding to the processed forms of

CDTA, CDTB, and CDTC by PCR with primer sets U714 and L1326, U1415 and L2188, and U2265 and L2801, respectively (Table 2). Each of them was placed in frame downstream of the His<sub>6</sub> tag sequence of the pET28a vector. After induction with IPTG, each construct yielded a considerable amount of fusion protein in recombinant *E. coli* HMS174 (47). These fusion proteins, designated HisCDTA, HisCDTB, and HisCDTC, respectively, were purified to homogeneity with an Ni-chelated TSKgel AF-chelate column and subsequent preparative SDS-PAGE (not shown). The purified samples were used for immunization of rabbits. The antisera thus obtained were used for detection of CDT proteins in *E. coli* XL-1 Blue(pTK3022) and *A. actinomycetemcomitans* Y4 cell lysates and in culture supernatants by Western blotting. As shown in Fig. 7, anti-HisCDTA, -HisCDTB, and -HisCDTC sera reacted with 24.5-, 29.0-, and 17.4-kDa proteins in the culture supernatant and lysate of *E. coli*(pTK3022). Similarly, anti-HisCDTA and anti-HisCDTC sera reacted with 24.5- and 17.4-kDa proteins in *A. actinomycetemcomitans* Y4 cell lysate and culture supernatant. On the other hand, anti-CDTB reacted with a 29.0-kDa protein in sonic lysate but not in culture supernatant of *A. actinomycetemcomitans* Y4. As shown in Fig. 7A, anti-HisCDTA reacted with additional bands of lower molecular masses in culture supernatant and cell lysate of *E. coli*(pTK3022) and *A. actinomycetemcomitans* Y4. These additional bands might represent proteolytic digests of CDTA, but the identities of those cross-reactive bands remain unknown. Anti-CDT sera were then assayed for neutralizing activity against CDT from *A. actinomycetemcomitans* lysate or culture supernatant. As shown in Fig. 8, a 300-fold dilution of anti-CDTC completely blocked CDT activity of cell lysate from *A. actinomycetemcomitans* Y4 against HeLa cells. Anti-HisCDTA showed a weaker but clear neutralizing effect. In contrast, anti-HisCDTB failed to show any neutralizing effect at 300-, 150-, and 75-fold dilutions. Similar neutralizing effects were observed with these antisera against culture supernatant of *A. actinomycetemcomitans* Y4 (not shown). These antisera at effective doses were able to block not only cytodistension but subsequent cell cytotoxicity and morphological changes in the nucleus (not shown).

## DISCUSSION

The production of a cytotoxic factor distinct from leukotoxin has been reported by several groups working on *A. actinomycetemcomitans*. This heat-labile factor, present in the culture supernatant, was shown to inhibit growth of human and murine fibroblasts (41) and human keratinocytes (15) and to have an immunosuppressive activity towards human T and B cells (43). A similar toxic factor has been reported to inhibit human gingival fibroblasts in the G<sub>2</sub> phase of the cell cycle (10). In addition, *A. actinomycetemcomitans* also possesses a cytotoxic or immunosuppressive factor(s) associated with its cell surface (16, 24, 50). In the present study, we have established that *A. actinomycetemcomitans* produces a new member of the CDT family, which is a previously unrecognized virulence factor of *A. actinomycetemcomitans*. Amino acid sequences deduced from cloned genes indicate that *A. actinomycetemcomitans* CDT is very similar to *H. ducreyi* CDT (Table 3). It was recently shown that the different *E. coli* CDTs (29) and the CDT of *Campylobacter jejuni* (51) block the cell cycle in G<sub>2</sub> phase. We confirmed unambiguously that *A. actinomycetemcomitans* CDT also induced cell cycle arrest in G<sub>2</sub>/M phase. Taken together, our results strongly suggest that CDT is the cytotoxic factor present in the culture supernatant and cell homogenate of *A. actinomycetemcomitans* Y4.

The CDTs are produced by a variety of bacterial genera and



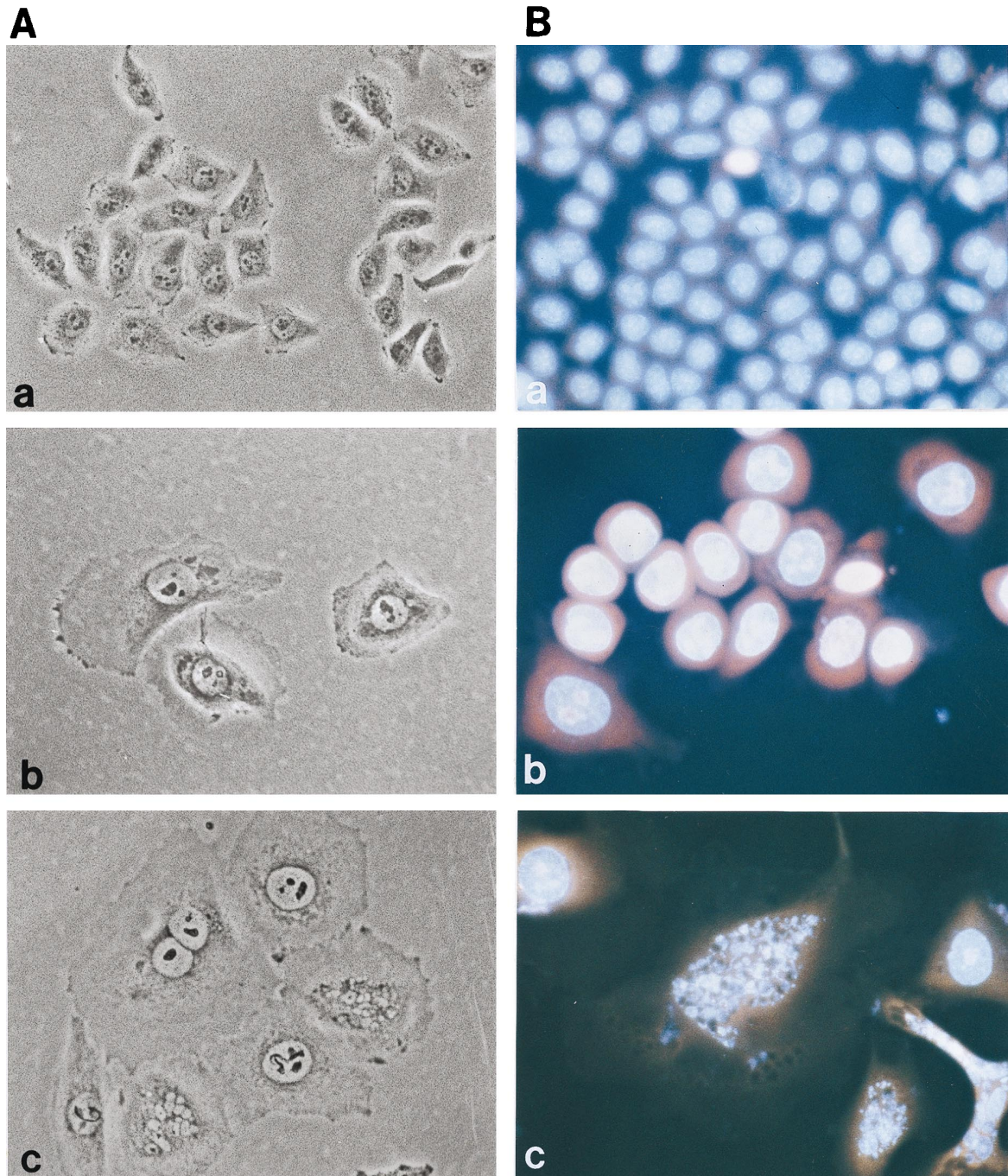


FIG. 5. Effect of *A. actinomycetemcomitans* Y4 CDT on morphology of cultured HeLa cells. Phase-contrast microscopy (A) and PI and Hoechst 33342 staining (B) of HeLa cells incubated with 20  $CD_{50}$  of a sterile sonic lysate of *E. coli*(pTK3022) for 3 days (b) or 4 days (c) are shown. (a) Control HeLa cells treated with a lysate of *E. coli* XL-1 Blue (5) for 4 days. Magnification,  $\times 200$ .

form a heterogeneous family of toxins with similar biological activities (7, 28, 29, 31, 32, 40). The term CDT was coined for activity that induces progressive cell distention and eventual cytotoxicity on cultured cells (12, 13). The CDT activity was first described for cell extracts of *Campylobacter* spp. and *E. coli* clinical isolates (12, 13). Now six CDTs have been identified and their genes have been cloned, in addition to *A. actinomycetemcomitans* CDT: three from *E. coli* (29, 31, 40), one from *C. jejuni* (32), one from *Shigella dysenteriae* (28), and one from *H. ducreyi* (7). CDTs are encoded by a cluster of

three genes, which are separated by a few nucleotides or slightly overlap. The *A. actinomycetemcomitans* *cdt* locus also encoded three proteins, CDTA, -B, and -C, which are all required for toxicity to the cells (Fig. 2). CDTA possesses a possible cleavage site for signal peptidase II, a lipoprotein-specific signal peptidase (4, 53). However, the significance of this amino acid sequence motif remains to be determined.

The origin of the *cdt* genes in *A. actinomycetemcomitans* remains unknown. The similarity of the amino acid sequences to those of the *H. ducreyi* CDTs together with the fact that



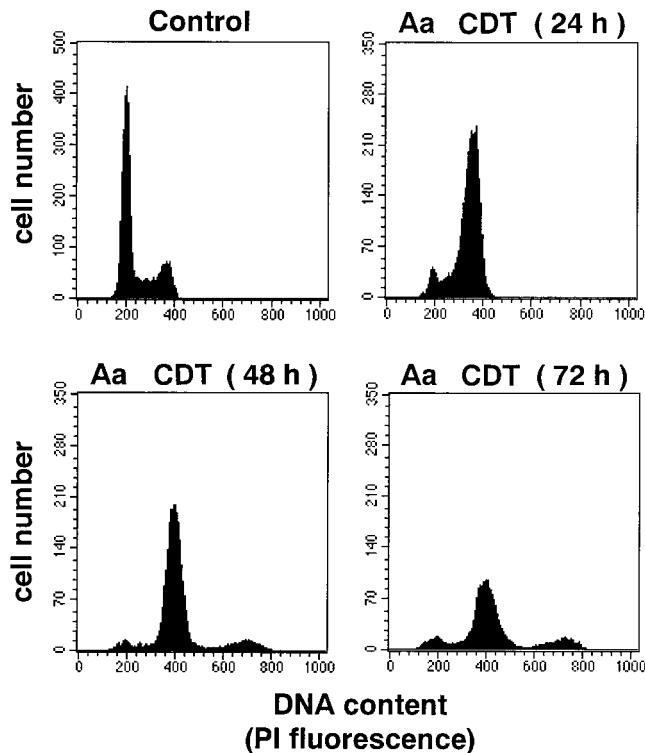


FIG. 6. Effect of *A. actinomycetemcomitans* Y4 (Aa) CDT on cell cycle pattern of HeLa cells. HeLa cells were incubated with 20  $CD_{50}$  of a sterile sonic lysate of *E. coli*(pTK3022) for the indicated times.

*Actinobacillus* and *Haemophilus* are closely related in evolutionary origin suggest that the *Actinobacillus* and *Haemophilus* *cdt* genes originated from the same ancestral genes. Sequence analysis suggested that *H. ducreyi* *cdt* was acquired as part of a transposon (7). For *E. coli*, insertion sequences and phagic elements were found upstream of the *cdtIII* locus (29). The presence, close to *A. actinomycetemcomitans* *cdt*, of both an ORF with a predicted product homologous to VagC/VapB and a DNA sequence homologous to an integrating plasmid of *H. influenzae* suggests that the *cdt* locus of *A. actinomycetemcomitans* is or was on a plasmid. Southern hybridization failed to show any signal suggesting that the *cdt* genes were still located on a plasmid. Nonetheless, our results further support the notion that the genetic determinants of CDTs have been transferred horizontally among bacterial species (29).

An assay for neutralizing activity against CDT of *A. actinomycetemcomitans* Y4 lysate showed that anti-CDTC serum had the strongest neutralizing effect (Fig. 8). This was in good agreement with previous observations that a neutralizing monoclonal antibody against *H. ducreyi* cytotoxin recognizes CDTC (7, 34). By contrast, anti-CDTA showed a weaker neutralizing effect, and anti-CDTB showed only slight inhibition of the CDT activity. It should be noted that Western analysis demonstrated that only CDTA and CDTC were present in the culture supernatant of *A. actinomycetemcomitans* Y4, while all CDT subunits were present in the sonic lysate of the bacteria (Fig. 8). It is possible that CDTB is a cell-bound protein and is involved in release of other CDTs from *A. actinomycetemcomitans* Y4, but further study is necessary to understand the secretion mechanism and function of *cdt* gene products.

The eukaryotic target of *A. actinomycetemcomitans* CDT is not known at present. Like the CDTs of *E. coli* and *C. jejuni*, CDT of *A. actinomycetemcomitans* induced in HeLa cells growth arrest at the  $G_2/M$  phase of the cell cycle. Recently, Comayras et al. (6) reported that CDT treatment causes HeLa cells to

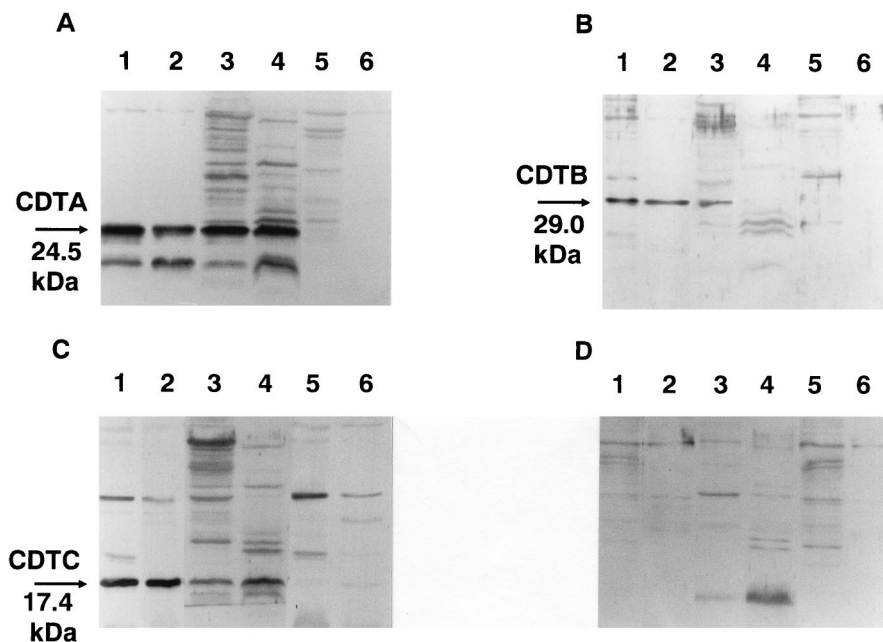


FIG. 7. Immunological detection of CDT in culture supernatant or sonic lysate of *A. actinomycetemcomitans* or recombinant *E. coli* carrying pTK3022. Western blotting was performed as described previously (49). Immunodetection was performed with antiserum against His-tagged CDTA (A), His-tagged CDTB (B), or His-tagged CDTC (C) or with control nonimmune serum (D). Lanes: 1, sonic lysate of *E. coli* XL-1 Blue(pTK3022); 2, culture supernatant of *E. coli* XL-1 Blue(pTK3022); 3, sonic lysate of *A. actinomycetemcomitans* Y4; 4, culture supernatant of *A. actinomycetemcomitans* Y4; 5, sonic lysate of *E. coli* XL-1 Blue(pUC19); 6, culture supernatant of *E. coli* XL-1 Blue(pUC19).

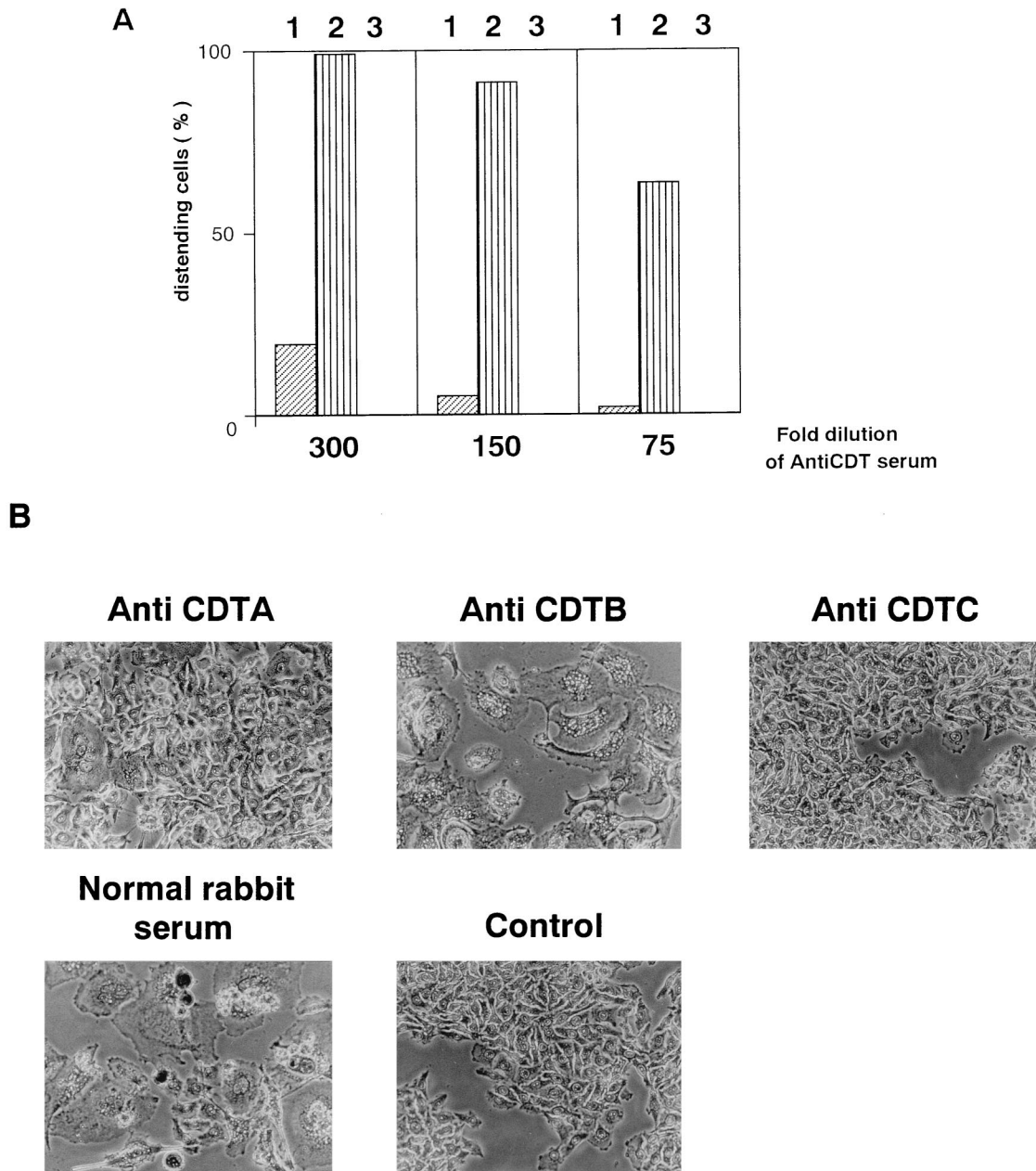


FIG. 8. Effect of anti-CDT serum on cytodistending activity of sterile sonic lysate of *A. actinomycetemcomitans* Y4. (A) HeLa cells were incubated with a sterile sonic lysate of *A. actinomycetemcomitans* Y4 (17  $CD_{50}$ ) in the presence of the indicated dilutions of anti-CDTA (bars 1), anti-CDTB (bars 2), and anti-CDTC (bars 3) for 4 days. The percentage of distended cells was calculated by counting the number of distended cells per 200 cells. (B) Phase-contrast microscopy of HeLa cells incubated with a sterile sonic lysate of *A. actinomycetemcomitans* Y4 (17  $CD_{50}$ ) in the presence of a 300-fold dilution of indicated antiserum for 4 days. Control, cells without treatment. Magnification,  $\times 150$ .

accumulate the inactive, tyrosine-phosphorylated form of CDC2. This result, recently confirmed by Whitehouse et al. with the CDT produced by *C. jejuni* (51), indicated that CDT treatment results in a failure to activate CDC2, which leads to cell cycle arrest in  $G_2$ . Both research groups suggest that the CDTs trigger a mechanism of cell cycle arrest by way of a DNA damage checkpoint system.

The role of *A. actinomycetemcomitans* CDT in the pathogenesis of periodontal diseases remains to be determined. *E. coli* and *S. dysenteriae* CDTs are implicated in inflammatory responses in bacterial infection in the gut (3, 12, 13, 27). As

suggested by previous reports of cytotoxic factors other than leukotoxin, cell death or growth arrest of fibroblasts may lead to a decrease in collagen synthesis, which will be manifested as a loss of collagen in periodontal disease. In this regard, elaboration of CDT by *A. actinomycetemcomitans* may be very relevant. Preliminary observations suggest that most clinical strains investigated possess the *cdt* gene cluster and produce CDTs extracellularly (unpublished results). Furthermore, CDT may function in concert with other virulence factors, such as leukotoxin and lipopolysaccharide, to induce tissue damage in the periodontal milieu. Recently, Kato et al. (18) reported that

*A. actinomycetemcomitans* infection induced apoptosis in a murine macrophage cell line in vitro. Another possible role of *A. actinomycetemcomitans* CDT could be suppression of immune cells targeting periodontal lesions. The antiproliferative activity of CDT could account for the pathogenesis of *A. actinomycetemcomitans* strains by blocking in vivo the expansion of lymphocytes, thus inhibiting the local immune response. Further study is clearly necessary to understand the true importance of this toxin in the pathogenesis of periodontal diseases.

#### ACKNOWLEDGMENTS

We thank the Research Facility, Hiroshima University School of Dentistry, for the use of their facilities.

This work was supported in part by a grant-in-aid for scientific research (09670282) from the Ministry of Education, Science, Sports and Culture of Japan. S.Y.P. is a recipient of a scholarship from the Ministère de l'Enseignement Supérieur et de la Recherche (France).

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Editor: J. T. Barbieri