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Molecular Characterizations of Cytolethal Distending Toxin Produced by *Providencia alcalifaciens* Strains Isolated from Patients with Diarrhea

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Cytolethal distending toxins (CDTs), which block eukaryotic cell proliferation by acting as inhibitory cyclomodulins, are produced by diverse groups of Gram-negative bacteria. Active CDT is composed of three polypeptides—CdtA, CdtB, and CdtC—encoded by the genes *cdtA*, *cdtB*, and *cdtC*, respectively. We developed a PCR-restriction fragment length polymorphism assay for the detection and differentiation of five alleles of *cdtB* (Cdt-I through Cdt-V) in *Escherichia coli* and used the assay to investigate the prevalence and characteristic of CDT-producing *E. coli* in children with diarrhea (A. Hinenoya et al., Microbiol. Immunol. 53:206–215, 2009). In these assays, two untypable *cdtB* genes were detected and the organisms harboring the *cdtB* gene were identified as *Providencia alcalifaciens* (strains AH-31 and AS-1). Nucleotide sequence analysis of the *cdt* gene cluster revealed that the *cdtA*, *cdtB*, and *cdtC* genes of *P. alcalifaciens* are of 750, 810, and 549 bp, respectively. To understand the possible horizontal transfer of the *cdt* genes among closely related species, the presence of *cdt* genes was screened in various *Providencia* spp. by colony hybridization assay, and the *cdt* gene cluster was found in only limited strains of *P. alcalifaciens*. Genome walking revealed that the *cdt* gene cluster of *P. alcalifaciens* is located adjacent to a putative transposase gene, suggesting the locus might be horizontally transferable. Interestingly, the CDT of *P. alcalifaciens* (PaCDT) showed some homology with the CDT of *Shigella boydii*. Whereas filter-sterilized lysates of strains AH-31 and AS-1 showed distention of CHO but not of HeLa cells, *E. coli* CDT-I exhibited distention of both cells. This activity of PaCDT was confirmed by generating recombinant PaCDT protein, which could also be neutralized by rabbit anti-PaCdtB antibody. Furthermore, recombinant PaCDT was found to induce G_2/M cell cycle arrest and phosphorylation of host histone H2AX, a sensitive marker of DNA double-strand breaks. To our knowledge, this is the first report showing that certain clinical *P. alcalifaciens* strains could produce variants of the CDTs compared.

Cytolethal distending toxin (CDT) was first discovered as a new type of toxin in *Escherichia coli* strains isolated from patient with diarrhea in 1987 (29). CDT has a unique activity, which differs from heat-labile enterotoxin (LT). Although LT causes only cell elongation, CDT causes not only cell elongation but also cell distention and blocking of eukaryotic cell cycle at G_2/M phase, leading to cell death. Since 1987, the presence of CDTs has been reported in various Gram-negative bacteria, such as *Aggregatibacter actinomycetemcomitans*, *Campylobacter* spp., *Escherichia albertii*, *Haemophilus ducreyi*, *Helicobacter* spp., and *Shigella* spp. (55). CDTs are composed of three polypeptides, namely, CdtA, CdtB, and CdtC, which form a complex structure needed for the toxin activity (55). CdtA and CdtC subunits bind the eukaryotic cell surface, followed by entry of the CdtB subunit into the cell (55). After entering the cell, the CdtB was transferred in the nucleus (55), and ultimately it causes DNA double-strand breaks by using its DNase I activity (15, 32). Therefore, CDT has recently been recognized as a new family of bacterial toxins, called genotoxin (37) or cyclomodulin (38).

In *E. coli*, at least five different types of EcCDTs have been reported thus far. EcCDT-I and EcCDT-II were initially identified in enteropathogenic *E. coli* (EPEC) strains isolated from patients with diarrhea (45, 46). EcCDT-III was discovered in an *E. coli* strain isolated from a calf with septicemia (43). EcCDT-IV was detected in pathogenic *E. coli* strains isolated from human or animal intestinal and extraintestinal sources (50), whereas EcCDT-V was identified in Shiga toxin-producing *E. coli* (STEC) or enterohemorrhagic *E. coli* (EHEC) strains (28). Among five different EcCDTs, it has been reported that the EcCDT-III gene is carried by a conjugative plasmid, called pVir (43), whereas EcCDT-IV and EcCDT-V genes are carried by the lambdoid and P2 phages, respectively (28, 51). Asakura et al. (5) has recently reported that EcCDT-I produced by certain strains of EPEC was encoded on an inducible lambdoid phage.

Although a number of studies regarding the isolation and characterization of CDT-producing bacteria from patients with diarrhea have been reported (2, 4, 9, 20, 24, 34, 39, 41), the role of CDT in human diseases, including diarrhea, has not yet been established. However, it has been demonstrated that shedding of *Helicobacter hepaticus cdt*-negative mutant was much shorter, and

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TABLE 1 Bacterial strains used in this study

Bacterium	Strain	Characteristic	Source or reference
P. alcalifaciens	AH-31	Clinical isolate (PaCDT)	24
	AS-1	Clinical isolate (PaCDT)	Diarrheal children (this study)
	F90-2004	Clinical isolate	ICDDR,B
	24717	Clinical isolate	ICDDR,B
	GTC2020	Clinical isolate	Purchased from Gifu University
	18H253	Clinical isolate	Osaka Prefectural Institute of Public Health
	18H399	Clinical isolate	Osaka Prefectural Institute of Public Health
	19H270	Clinical isolate	Osaka Prefectural Institute of Public Health
	P2556	Clinical isolate	Diarrheal adult (unpublished)
	RME362	Human isolate	Specimen from regular medical examination (unpublished)
P. rettgeri	GTC1263 (ATCC 29944)	Unknown	Purchased from Gifu University
	P2234	Clinical isolate	Diarrheal children (unpublished)
	P2253	Clinical isolate	Diarrheal children (unpublished)
	P2312	Clinical isolate	Diarrheal children (unpublished)
	P2536	Clinical isolate	Diarrheal children (unpublished)
	RME220	Human isolate	Specimen from regular medical examination (unpublished)
P. rustigianii	GTC1504 (ATCC 33673)	Human isolate	Purchased from Gifu University
	RME3	Human isolate	Specimen from regular medical examination (unpublished)
P. heimbachae	GTC1501 (ATCC 35613)	Penguin isolate	Purchased from Gifu University
P. stuartii	GTC1444 (ATCC 29914)	Human isolate	Purchased from Gifu University
E. coli	GB1371	Clinical isolate (EcCDT-I)	41
	C600	NA[a]	Laboratory strain (C. Sasakawa)
	BL21(DE3)	NA	Laboratory strain (Promega)
	BL21(DE3)	With pET28a	This study
	BL21(DE3)/TAS-1	With pAS-1 (rPaCdtB)	This study
	BL21(DE3)/TAS-2	With pAS-2 (rPaCDT)	This study

[a] NA, not applicable.

there was also mild inflammation of intestine compared to that of its isogenic wild-type strain (57). Recombinant *S. dysenteriae* CDT could cause diarrhea in the suckling mouse model (40). Furthermore, coadministration of *H. ducreyi* and purified *H. ducreyi* CDT could induce more severe inflammation than *H. ducreyi* alone (54). It is noteworthy that high amount of CDT-producing *E. coli* (CTEC) strains have been isolated from patients with bloody diarrhea in India (41). CTEC was also isolated from patients with bloody diarrhea as a sole pathogen in Japan (23). It has been reported that EcCDT-V genes in EHEC O157:H7 strains were significantly more frequent in isolates from patients with diarrhea than in isolates from asymptomatic carriers (18) and that *cdt* in *eae* (*E. coli* attaching and effacing)-negative STEC was significantly more frequent in patients with hemolytic-uremic syndrome and in patients with diarrhea than in asymptomatic carriers (7). These data suggest that CDT could possibly be a virulence factor and may contribute to persistence of infection or it could enhance pathogenicity in host. To elucidate the relevance of CTEC in diarrhea, we developed a PCR-restriction fragment length polymorphism (RFLP) assay for the detection and differentiation of *cdt* genes in *E. coli* and examined the prevalence and characteristics of CTEC among children with diarrhea in Japan (24). Untypeable *cdtB* genes were detected directly from two stool samples of patients with diarrhea by the PCR-RFLP assay, and the bacteria harboring these untypeable *cdtB* genes were isolated and identified as *Providencia alcalifaciens* (24; unpublished data).

In the present study we attempted to analyze the *cdt* gene cluster and its flanking region in the genome of *P. alcalifaciens*. To examine the possible horizontal transfer of the *cdt* gene cluster among closely related species, the distribution of *cdt* genes in various *Providencia* spp. was also checked. In addition, the biological activity and possible mechanism of action of these CDTs produced by the *P. alcalifaciens* strains were examined and discussed.

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MATERIALS AND METHODS

Bacterial strains and growth condition. The bacterial strains used in the present study are listed in Table 1. Two *P. alcalifaciens* isolated from children with diarrhea during surveillance of *cdt* gene harboring *E. coli* and identified by biochemical test using API 20E and adonitol and galactose utility tests (24, unpublished) were examined. Furthermore, eight *P. alcalifaciens* strains, six *P. rettgeri* strains, two *P. rustigianii* strains, one *P. heimbachae* strain, and one *P. stuartii* strain were also investigated for the presence of *cdt* genes. Bacteria were grown aerobically in Luria-Bertani (LB) medium (Becton Dickinson, Franklin Lakes, NJ), in brain heart infusion (BHI) medium (Becton Dickinson) or on LB agar (Becton Dickinson) containing 30 μg of kanamycin (Nacalai Tesque, Inc., Kyoto, Japan)/ml when appropriate.

Isolation of *Providencia* spp. Rectal swabs collected from patients with diarrhea were plated on polymyxin-mannitol-xylitol medium for

Providencia (PMXMP) agar (56). In addition, stool specimens obtained from a regular medical examination of 3- to 9-year-old children (kindergarten and primary school) were also screened by plating on PMXMP agar. Suspected bacterial colonies were isolated and identified as *Providencia* spp. by the API 20E system, and adonitol and galactose utility tests.

Colony hybridization assay for detection of *cdt* genes. To examine the presence of *cdt* genes in *P. alcalifaciens* strains and in other *Providencia* spp., including *P. rettgeri*, *P. rustigianii*, *P. heimbachae*, and *P. stuartii*, the *cdtB* DNA fragment was generated by PCR using the Cdt-Bcomu and Cdt-Bcomd primers, and the fragment was used as a probe in colony hybridization assay (24). Strain AH-31 was always used as a positive control.

PCR. PCR for the detection of *cdtB* gene was performed as described previously (24).

Nucleotide sequence analysis. To determine the nucleotide sequence of the *cdt* gene cluster, PCR products of *cdtB* gene and its flanking region were sequenced either by a standard method or by genome walking (6). Briefly, PCR product was purified by QIAquick PCR products purification kit (Qiagen, GmbH, Hilden, Germany), and the nucleotide sequence of the PCR product was determined by using a BigDye terminator cycle sequencing kit on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) essentially as described by the manufacturer. Synthetic primers were designed on the basis of obtained sequence and genome walking was performed as described previously (6). Nucleotide and amino acid sequences were analyzed and compared by using GenBank, DDBJ (the DNA Data Bank of Japan), and DNASIS software (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). Dendrogram analysis was performed by using the software CLUSTAL W of MegAlign (DNASTAR, Inc., Madison, WI).

Preparation of recombinant proteins. The *P. alcalifaciens cdt* and *cdtB* genes (Pa*cdt* and Pa*cdtB*) were amplified from the genomic DNA of *P. alcalifaciens* strain AH-31 by PCR using the primer set PacdtABC-F (5′-ATATGGATCCATGAATAATAAACGCACAT-3′) and PacdtABC-R (5′-ATATCTCGAGTTTAAATAACGGGTGACTC-3′) and the primer set PacdtB-F (5′-GAGAGGATCCGTGTTTTTATCGTTTTACGC-3′) and PacdtB-R (5′-GAGACTCGAGTTTACCTTCTGAATACGCC-3′), respectively. PCR was carried out in a 50-μl reaction mixture for each tube containing 2.5 μl of DNA template, 1× ExTaq PCR buffer (Takara Bio, Inc., Shiga, Japan), 0.2 mM deoxynucleoside triphosphate mixture, 0.5 μM concentrations of each primer set, and 1.25 U of ExTaq polymerase (Takara Bio, Inc.). DNA template was prepared from an overnight culture, which was diluted 10-fold in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and boiled for 10 min, followed by centrifugation at 12,000 × *g* at 4°C for 5 min. The PCR conditions were optimized as an initial denaturation of 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 90 s or 60 s at 72°C, with a final extension step for 5 min at 72°C in GeneAmp PCR System 9700 (Perkin-Elmer, Waltham, MA). Each PCR product was digested with BamHI and XhoI and ligated into pET-28a (pAS-1 for Pa*cdtB*, pAS-2 for Pa*cdt*), and pAS-1 and pAS-2 were transformed into *E. coli* BL21(DE3) (strain TAS-1 with pAS-1 and strain TAS-2 with pAS-2). *E. coli* strain BL21(DE3) with pET-28a was similarly prepared and used as a vector control. Recombinant PaCdtB (rPaCdtB) was purified from crude rPaCdtB expressed in *E. coli* strain TAS-1. Briefly, *E. coli* strain TAS-1 was grown at 37°C overnight in LB broth containing kanamycin (30 μg/ml). The culture was diluted 1:100 in the fresh medium and incubated at 37°C until the optical density at 600 nm of the culture reached 0.6. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.1 mM, and the culture was further incubated at 18°C for 16 h with vigorous shaking. The bacterial cells were then collected by centrifugation at 6,000 × *g* at 4°C for 15 min. The cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 50 mM imidazole and then sonicated using an Astrason ultrasonic processor (Heat-System Ultrasonics, Farmingdale, NY). The lysates were centrifuged at 15,000 × *g* at 4°C for 15 min, and the supernatants were collected and used for further purification. The rPaCdtB was purified by using an Ni-Sepharose column (GE Healthcare UK, Ltd., Buckinghamshire, England). The purity of the rPaCdtB was confirmed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) (31). For the preparation of crude rPaCDT, *E. coli* strain TAS-2 was cultured at 37°C overnight with vigorous shaking. The culture was sonicated as described above, the lysates were centrifuged at 12,000 × *g* at 4°C for 5 min, and the supernatants were filtrated using a 0.22-μm-pore-size filter (Asahi Glass Co., Ltd., Tokyo, Japan). The filter-sterilized lysate of *E. coli* strain BL21(DE3)/pET28a was similarly prepared as a vector control. The filtrate was either directly used for cytotoxicity assay or used for suckling mouse assay after concentration by ammonium sulfate precipitation. For ammonium sulfate precipitation, solid ammonium sulfate was first added to 40% saturation, and then the precipitate was removed by centrifugation at 20,000 × *g* at 4°C for 20 min. Finally, the supernatant solution was brought to 60% saturation with respect to ammonium sulfate. After centrifugation at 20,000 × *g* at 4°C for 20 min, the precipitate was dialyzed against phosphate-buffered saline (PBS; pH 7.4) until ammonium sulfate was completely removed. The crude rPaCDT was then used for suckling mouse assay. The endotoxin content of rPaCDT was determined by using the ToxinSensor endotoxin detection system (GenScript USA, Inc., Piscataway, NJ) and confirmed to be similar to the preparation from negative control [*E. coli* BL21(DE3) harboring pET28a].

Preparation of antisera against rPaCdtB. Purified rPaCdtB was immunized against 8-week-old male New Zealand White rabbit (Oriental Yeast Co., Ltd., Tokyo, Japan). Briefly, 300 μg of purified rPaCdtB was injected into four sites: subcutaneously into the shoulders and intramuscularly into the thighs every 2 weeks interval with Freund complete adjuvant (Becton Dickinson) first and subsequently with Freund incomplete adjuvant (Becton Dickinson) for 8 weeks. The rabbits were anesthetized with ketamine (35 mg/kg [body weight]) and xylazine (5 mg/kg [body weight]), blood was collected, and serum was obtained by centrifugation at 6,000 × *g* for 10 min.

Determination of titer. The titer was determined by using an Ouchterlony double gel diffusion test as described previously (58). Briefly, the double gel diffusion test was carried out with 1.2% Noble agar (Difco) in 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. Each sample was applied into a hole, and the plate was placed in a humidified chamber at room temperature for about 16 to 24 h. The plate was then washed extensively with a solution of 0.4% NaCl and 0.4% sodium borate and dried. The plate was stained with 0.5% Coomassie brilliant blue (CBB) dissolved in a solution of 50% methanol, 10% acetic acid, and 40% water and destained with the same solution without CBB. The antibody titer was defined as the highest dilution of serum that yielded a visible precipitation line by CBB staining.

Western blotting. *P. alcalifaciens* strains AH-31 and AS-1 were cultured at 37°C for 16 h in BHI medium, respectively. A 1-ml portion of the culture was centrifuged at 6,000 × *g* for 10 min, and the cells were resuspended and sonicated in 200 μl of PBS for 1 min on ice using a handy sonicator UR-20P (Tomy Seiko Co., Ltd., Tokyo, Japan). Cell lysates were separated by SDS-15% PAGE as described above. The proteins were blotted to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using a Trans-Blot SD semidry electrophoretic transfer cell essentially as described by the manufacturer (Bio-Rad) and probed with anti-rPaCdtB antiserum, followed by goat anti-rabbit IgG tagged with horseradish peroxidase (HRP) as a secondary antibody (Invitrogen, Carlsbad, CA). Color development was performed with 4CN-PLUS (Perkin-Elmer) at room temperature.

Cell culture. HeLa, Vero, Int407, HEp2, and Caco-2 cells were cultured in minimum essential medium (MEM; Invitrogen). CHO, Y-1, or NIH/3T3 was cultured either in MEM-α (Invitrogen), Ham F-12 (Invitrogen), or Dulbecco's modified Eagle medium (Invitrogen), respectively. All media contained 10% fetal bovine serum (Invitrogen) and 1% antibiotic, including antimycotic (×100) liquid (penicillin G sodium [10,000 U/ml], streptomycin sulfate [10,000 U/ml], and 25 μg of amphotericin

B/ml as Fungizone in 0.85% saline [Invitrogen]). In addition, 1% nonessential amino acids solution (×100; Invitrogen) was added to the MEM for Caco-2 cells. The cells were cultured at 37°C under 5% CO_2 in air.

Cytotoxicity assay. *P. alcalifaciens* strains (AH-31 and AS-1) harboring Pa*cdt* genes (Table 1) were cultured at 37°C for 16 h in an appropriate medium, and the culture was sonicated as described above. The lysates were passed through a sterile disposable filter with a 0.22-μm pore size, and filter-sterilized bacterial lysates were examined for the ability to cause the distension and death of CHO, HeLa, Vero, HEp2, Int407, Caco-2, Y-1, and NIH/3T3 cells. Filter-sterilized lysates of *E. coli* strain TAS-2 (rPaCDT) were also included. Filter-sterilized lysates of *E. coli* strains GB1371 (EcCDT-I) and C600 were used as positive and negative controls, respectively. The cells were seeded at a density of 5×10^3 cells in a 96-well plate (Asahi Glass Co., Ltd.). After 24 h of incubation, 20 μl of 2-fold serially diluted crude PaCDT was added. For the neutralization assay, 20 μl of 2-fold serially diluted rabbit anti-rPaCdtB serum, as well as preimmune serum, was added with filter-sterilized bacterial lysates into the cell culture. Cell morphology was observed after 72 h of incubation under microscopy. A neutralizing titer was defined as the highest dilution of serum that inhibits the 50% cytotoxicity caused by PaCDT.

Plasmid isolation. Plasmid DNA was extracted from 100 ml of overnight bacterial culture by the alkaline lysis method (8) and electrophoresed in 0.7% agarose.

Analysis of cell cycle inhibition. To measure cell cycle arrest induced by PaCDT, HeLa or CHO cells were seeded at density of 2×10^5 cells in a 25-cm^2 flask (Corning, NY). After 24 h of incubation, 1 ml of a 5-fold serially diluted filter-sterilized lysate of *P. alcalifaciens* strain AH-31 or AS-1 (PaCDT) or *E. coli* TAS-2 strain (rPaCDT) was added to the flask. Filter-sterilized lysates of *E. coli* strains GB1371 (EcCDT-I) and C600 were used as positive and negative controls, respectively. After 24 h of incubation, the medium was replaced, and incubation continued for another 24 h. Cells were collected and fixed for 1 h on ice with 70% ethanol. The cells were then stained with propidium iodide (50 μg/ml) in PBS containing 0.25 mg of RNase A (Sigma, St. Louis, MO)/ml at 4°C for 20 min in the dark. For each flask, 10^4 cells were analyzed by using FACSCalibur (Becton Dickinson). Cell cycle analysis was performed using BD CellQuest Pro software (Becton Dickinson).

Fluorescence microscopy. CHO cells (10^4) were seeded on a glass slide (Nalge Nunc International, Rochester, NY) and allowed to adhere for 24 h. The cells were incubated with the filter-sterilized bacterial lysate of strain AH-31 for 16 h. Intoxicated cells were fixed in 3.7% formaldehyde for 10 min, treated with ice-cold methanol for 20 min at −30°C, and then treated with 0.5% Triton X-100 for 20 min. Cells were stained with Alexa Fluor 546-conjugated phalloidin (Invitrogen). For immunostaining, the cells were blocked in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin for 1 h and then treated with Alexa Fluor 488-conjugated anti-phospho-histone H2AX (phosphor-Ser139) antibodies (Cell Signaling Technology, Danvers, MA) at 4°C overnight.

Purification of recombinant cholera toxin. Recombinant cholera toxin (rCT) used as a positive control for suckling mice assay was purified as described previously (52).

Suckling mouse assay and diarrhea score. The enterotoxic activity of CDT produced by *P. alcalifaciens* was examined by using suckling mouse assay and diarrhea score was calculated as described previously (40). Either rPaCDT, which showed a high titer (50% cytotoxic dose [CD_{50}] = 2,560) to CHO cell cytotoxicity, or 10^{10} CFU of live bacteria such as *P. alcalifaciens* strains AH-31 and AS-1 and *E. coli* C600 (negative control), respectively, was given orally to each mouse.

Nucleotide sequence accession number. The nucleotide sequences of *cdt* genes and its flanking region of *P. alcalifaciens* strains AH-31 and AS-1 have been registered in DDBL under accession numbers AB583184 and AB583185, respectively.

Statistical analysis. To compare the effect of CDT-producing strains [strains AH-31, AS-1, GB1371, and BL21(DE3)/TAS-2] and nontoxigenic control strains [strains C600 and BL21(DE3)] on CHO cells (G_1 and G_2/M), statistical analysis was performed using the Student *t* test. A *P* value of <0.05 was considered significant ($n = 3$).

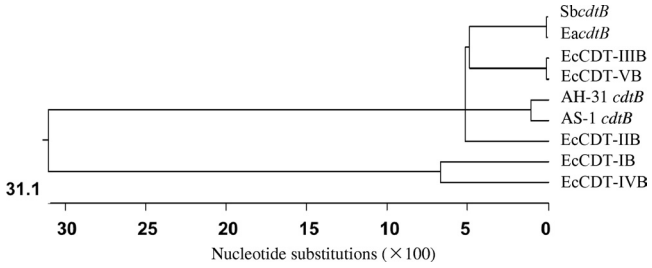

FIG 1 Dendrogram analysis of *cdtB* genes. Dendrogram analysis was performed using CLUSTAL W of MegAlign. Genes: AH-31 *cdtB*, *cdtB* of *P. alcalifaciens* strain AH-31 (AB583184); AS-1 *cdtB*, *cdtB* of *P. alcalifaciens* strain AS-1 (AB583185); EcCDT-IB (U03293), EcCDT-IIB (U04208), EcCDT-IIIB (U89305), EcCDT-IVB (AY578329), EcCDT-VB (AJ508930), Ea*cdtB* (AT696755), Sb*cdtB* (AT696753).

RESULTS

Nucleotide sequence of the *P. alcalifaciens cdt* genes. We sequenced 12,064 bp, including the *cdt* genes and its flanking region of *P. alcalifaciens* strains AH-31 and 2,186 bp of the *cdt* genes of the strain AS-1. These two DNA fragments contained three open reading frames (ORFs) termed Pa*cdtA* (750 bp, 249 amino acids [aa]), Pa*cdtB* (810 bp, 269 aa), and Pa*cdtC* (549 bp, 182 aa). Dendrogram analysis indicated that Pa*cdtB* is highly homologous to the *Shigella boydii cdtB*, *E. albertii cdtB*, and EcCDT-IIIB and EcCDT-VB genes (Fig. 1). Furthermore, ORFs 1 to 10 were found in the upstream region of Pa*cdt* genes in the AH-31 strain, although ORFs 14 to 23 were found in the downstream region, as shown in Fig. 2 and Table 2. Detailed data regarding percent G+C (%GC) content and homologous proteins are summarized in Table 2 (10, 12, 14, 25, 30, 42, 44, 49, 53, 59). Although genes related to prophage were not found and the functions of some gene products are unknown, genes partly homologous to transposase and the IS element were found in the flanking region as shown in Table 2 (14, 42, 49). In addition, no plasmid was found in *P. alcalifaciens* strains AH-31 and AS-1. These data indicated that the Pa*cdt* genes in AH-31 strain are most likely located in the chromosome and probably acquired by horizontal gene transfer mechanism through phage(s) or transposon(s).

The homology of the deduced amino acid sequences of the PaCdtA, PaCdtB, and PaCdtC proteins of two *P. alcalifaciens* strains was 96.4, 97.8, and 97.3%, respectively. The deduced amino acid sequences of PaCDT of the strain AH-31 were highly homologous to the CDT of *S. boydii* (accession no. AY696753) with 92.4, 94.8, and 90.2% identities for CdtA, CdtB, and CdtC, respectively, followed by *E. albertii* CDT (AY696755: CdtA, 91.6%; CdtB, 94.8%; CdtC, 89.6%), EcCDT-II (U04208: CdtA, 89.6%; CdtB, 93.7%; CdtC, 85.5%), EcCDT-III (U89305: CdtA, 89.2%; CdtB, 94.4%; CdtC, 87.4%), and EcCDT-V (AJ508930: CdtA, 89.2%; CdtB, 94.4%; CdtC, 88.5%), and homologous to other CDTs reported in *A. actinomycetemcomitans* CDT (AB011405), EcCDT-I (U03293), EcCDT-IV (AY578329), *C. jejuni* CDT (U51121), *C. coli* CDT (AB182109), *C. fetus* CDT (AB211058), *H. ducreyi* CDT (U53215), *H. hepaticus* CDT (AF163667), and *S. dysenteriae* CDT (55) (CdtA, 16.8% to 35.6%; CdtB, 45.2% to 55.6%; CdtC, 18.6% to 32.2%). The putative amino acid residues

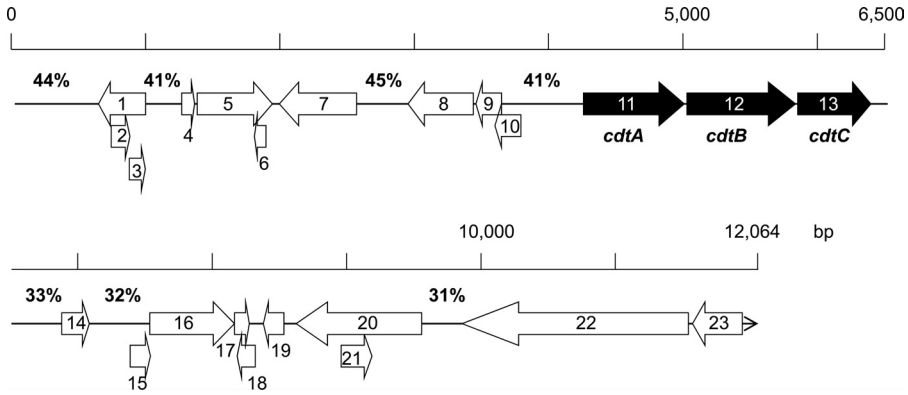

FIG 2 Schematic representation of the Pa*cdt* genes and its flanking regions of the strain AH-31. Closed and open arrows indicate Pa*cdt* genes and ORFs, respectively, located in the flanking region. The number indicated above each bar represents the %GC content of a noncoding region.

(H154, G191-N194, D229, and S259-V264) important for DNase I activity were perfectly conserved in PaCdtB. The nuclear localization signals (NLS1 and NLS2) detected in EcCDT-II were also almost conserved in PaCdtB, except for two amino acid substitutions in each of the regions (NLS1, A198D and R210N; NLS2, F262Y and S267F). The $RR(X)_{10\text{-}20}RR$ motif (33) in NLS2 was found to be completely conserved, suggesting that PaCDT may enter into the nucleus for its genotoxic activity.

TABLE 2 Characteristics of the ORFs of the Pa*cdt* gene and its flanking regions

ORF[a]	Gene coordinates and direction	Gene product size (aa)	GC%	Related bacterial proteins			Source or reference
				Product(s) and origin	GenBank accession no.	BLAST E-value (identity), %	
1	651←1001	117	47.86	Transposase (fragment), *Xenorhabdus nematophila* ATCC 19061	FN667742	4e–19 (64/110), 58%	Direct submission
2	745→885	47	49.65	Transposase (fragment), *Xenorhabdus nematophila* ATCC 19061	FN667742	7e–05 (22/39), 56%	Direct submission
3	882→1004	41	46.34	No hits found			
4	1270→1368	33	36.36	Unknown, *Comamonas testosteroni* PtL5	AF076997	0.24 (15/22), 68%	Direct submission
5	1387→1947	187	34.22	Unknown, *Photorhabdus luminescens* subsp. *laumondii* TTO1	BX571866	8e–65 (126/187), 67%	53
6	1812←1898	29	42.53	Putative DNA-binding protein, *Proteus mirabilis* HI4320	AM942759	8e–06 (24/28), 85%	42
7	1996←2574	193	43.01	Transposase, *Salmonella enterica* subsp. *salamae* serovar Sofia	FJ496648	7e–86 (154/192), 80%	Direct submission
8	2957←3445	163	47.65	Putative exported protein, *Citrobacter rodentium* ICC168	FN543503	1e–27 (55/100), 55%	44
9	3464←3655	64	44.27	Transposase, *Proteus mirabilis* HI4320	AM942759	5e–24 (54/67), 80%	42
10	3604←3801	66	41.92	Putative putative IS element transposase, *Proteus mirabilis* HI4320	AM942759	3e–10 (23/32), 71%	42
11*	4264→5013	750	42.80	Cytolethal distending toxin A, *Shigella boydii* strain K-1	AY696753	92%	25
12*	5034→5843	810	42.10	Cytolethal distending toxin B, *Shigella boydii* strain K-1	AY696753	95%	25
13*	5858→6406	549	37.34	Cytolethal distending toxin C, *Shigella boydii* strain K-1	AY696753	90%	25
14	6884→7090	69	29.47	Replication initiator and transcription repressor, *Pantoea stewartii* subsp. *stewartii*	L42524	3e–16 (47/94), 50%	19
15	7390→7542	51	41.83	Unknown, *Escherichia coli* strain CB853	FM210347	0.044 (22/50), 44%	10
16	7539→8168	210	45.40	Hypothetical protein, *Enterobacter cloacae*	AY780889	4e–78 (142/205), 69%	59
17	8168→8281	38	51.75	Transposase, *Escherichia coli* strain BEN2908	AY857617	1e–09 (30/41), 73%	14
18	8186←8323	46	44.93	Hypothetical ORF in IS*2*, *Klebsiella pneumoniae*	AY378100	0.020 (24/47), 51%	12
19	8384←8542	53	35.85	Transposase, *Clostridium difficile* CD196	FN538970	1.7 (15/34), 44%	49
20	8629←9567	313	37.49	EspG protein, *Escherichia coli* strain 71074	GQ338312	2e–44 (104/314), 33%	Direct submission
21	8960→9190	77	37.66	Pyridine nucleotide-disulfide oxidoreductase, *Listeria monocytogenes* HCC23	CP001175	32.7 (17/50), 34%	Direct submission
22	9861←11549	563	38.90	Protein tyrosine phosphatase SptP, *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain SL1344	U63293	9e–82 (190/559), 33%	30
23	11579←1195	124	31.45	Chaperone protein SicP, *Salmonella enterica* subsp. *salamae* serovar Sofia	FJ496648	2e–16 (43/93), 46%	Direct submission

[a] *, Results obtained from the CLUSTAL W method.

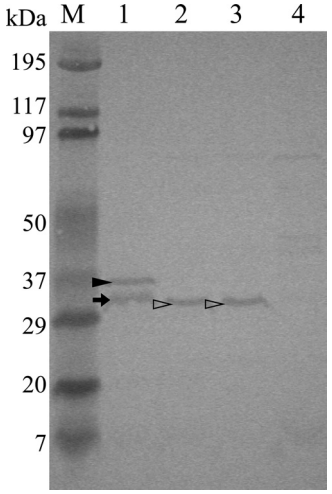

FIG 3 Detection of PaCdtB of wild-type *P. alcalifaciens* by Western blotting. Whole-cell lysate of *P. alcalifaciens* was separated by SDS-PAGE (15%), transferred into a PVDF membrane, and probed with rabbit anti-rPaCdtB antiserum, followed by treatment with goat anti-rabbit IgG tagged with HRP as a secondary antibody. Color development was performed with 4CN-PLUS. Lanes: M, prestained SDS-PAGE broad-range marker (Bio-Rad);1, purified rPaCdtB; 2, bacterial lysate of AH-31 (*P. alcalifaciens*); 3, bacterial lysate of AS-1 (*P. alcalifaciens*); 4, bacterial lysate of *E. coli* strain BL21(DE3) carrying empty vector pET28a. The closed and open arrowheads and the arrow indicate rPaCdtB, PaCdtB, and degraded product of rPaCdtB, respectively. The experiment was carried out at least thrice.

Distribution of *cdt* genes among *Providencia* spp. To examine the distribution of *cdt* genes among *Providencia* spp., 18 strains belonging to the genus *Providencia*, including eight *P. alcalifaciens* strains, six *P. rettgeri* strains, two *P. rustigianii* strains, one *P. heimbachae* strain, and one *P. stuartii* strain, were tested using the *cdtB* gene as a probe by colony hybridization assay. The probe was able to detect only a single strain of *P. alcalifaciens*, and further analysis revealed that the strain harbors a truncated *cdt* gene cluster, which was the most likely reason for the absence of CDT activity in this strain.

Expression of recombinant PaCDT. To confirm whether *P. alcalifaciens* strains AH-31 and AS-1 produced PaCDTs, we attempted to raise an antibody against PaCdtB for Western blotting and cytotoxic assay. For this purpose, a Pa*cdtB* gene was cloned and expressed in *E. coli* as rPaCdtB with His tag (see the details in Materials and Methods). Purified rPaCdtB was used to immunize rabbits and antiserum against rPaCdtB was successfully obtained. Western blotting revealed that antiserum against rPaCdtB was specific for PaCdtB (Fig. 3). Two bands (of about 35 and 33 kDa), which seem to be intact and degraded products of rPaCdtB, respectively, were obtained in lane 1 (rPaCdtB), while only one band (about 32 kDa) was obtained in lanes 2 and 3 with lysates of *P. alcalifaciens* strains AH-31 and AS-1, respectively. However, no reactive bands were obtained from the lysate of *E. coli* strain BL21(DE3) carrying the empty vector pET28a, indicating that *P. alcalifaciens* strains AH-31 and AS-1 produced PaCdtB and antibody against rPaCdtB was specifically reactive to the protein.

Genotoxic activity of PaCDT. Since production of PaCdtB was confirmed in both *P. alcalifaciens* strains AH-31 and AS-1, we further examined whether biologically active CDT was produced by these strains. Filter-sterilized lysates of both *P. alcalifaciens* strains AH-31 (Fig. 4C) and AS-1 (Fig. 4D) induced cell distention on CHO cells. Although filter-sterilized lysate of *E. coli* strain GB1371 (EcCDT-I) used as a positive control showed cell distention in both CHO (Fig. 4B) and HeLa (data not shown) cells, the filter-sterilized lysates of *P. alcalifaciens* strains AH-31 and AS-1 (PaCDT) did not show any morphological changes of HeLa cells (data not shown). Since the activities of PaCDT and EcCDT-I to CHO and HeLa cells were different, an additional six cell lines, namely, Vero, HEp2, Int407, Caco-2, Y-1, and NIH/3T3, were also tested to examine any such differential activities by these two

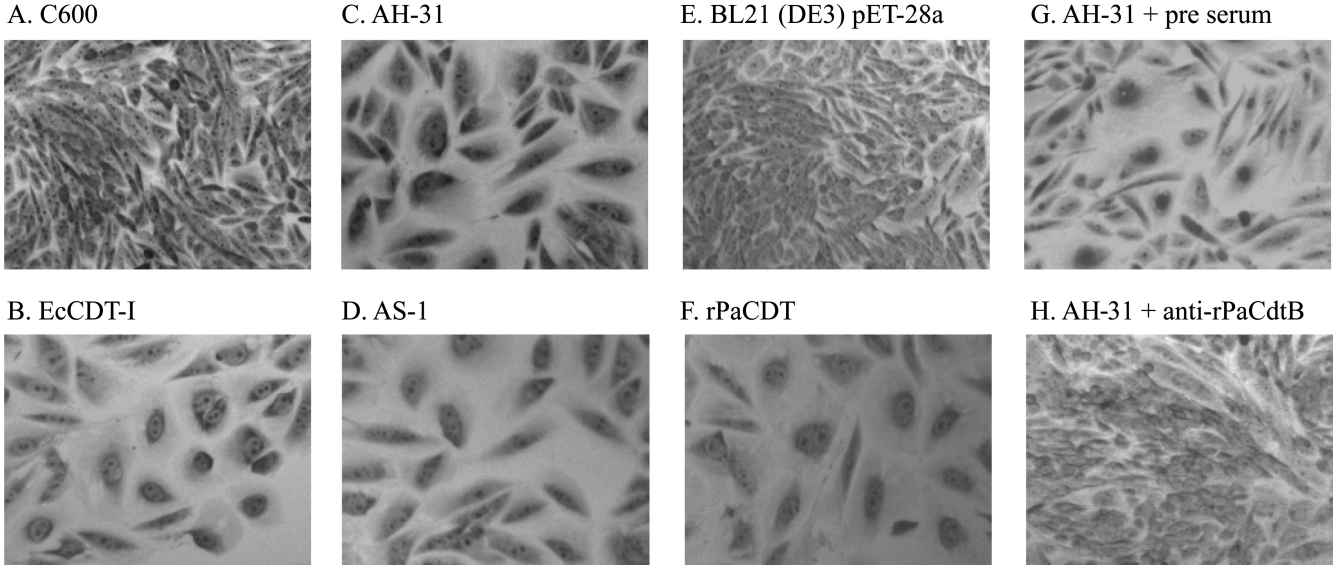

FIG 4 Cytotoxic effect of CDT produced by *P. alcalifaciens* on CHO cells. Whereas alteration of cellular morphology was not apparent at 72 h after exposure of the filter-sterilized samples from *E. coli* strain C600 (A) and *E. coli* strain BL21(DE3) carrying pET-28a as a vector control (E) to CHO cells, cytoplasmic distension was apparent by the filter-sterilized samples from *E. coli* strain GB1371 (EcCDT-I) (B), *P. alcalifaciens* strains AH-31 (C) and AS-1 (D), *E. coli* strain TSA-1 (rPaCDT) (F), and *P. alcalifaciens* strain AH-31 with preimmunization serum (G). The CDT activity of *P. alcalifaciens* strain AH-31 was neutralized in the presence of anti-rabbit rPaCdtB (H). The experiment was performed at least thrice.

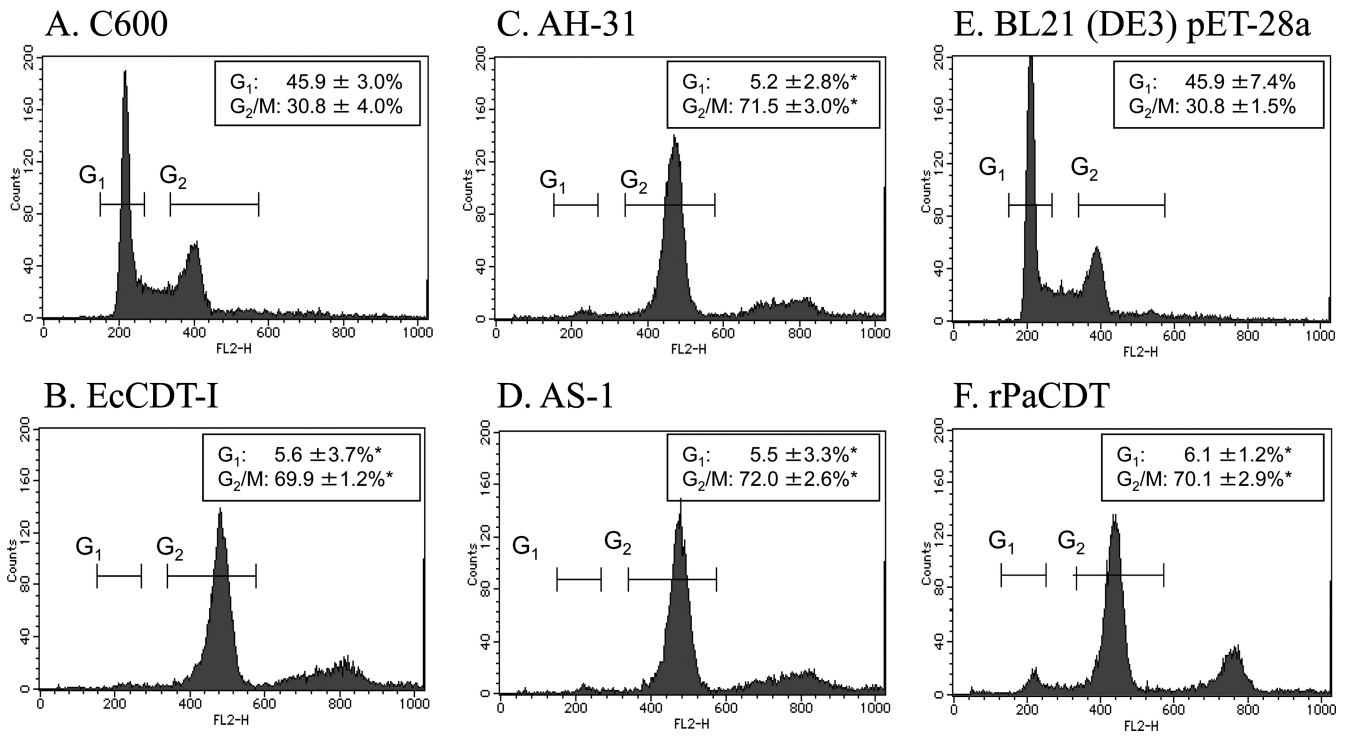

FIG 5 Analysis of CHO cell cycle after treatment with various preparations. The cell cycle distribution of 10,000 cells was determined by flow cytometry, and representative results are shown. The average percentages and the standard deviations of cells in each cell cycle phase calculated with three independent experiments are indicated. CHO cells were unaffected when exposed to lysates of nontoxigenic control *E. coli* strain such as C600 (A) or BL21(DE3) carrying the empty vector pET-28a (E) cells, but the cells were blocked in the G_2/M cell cycle phase when they were exposed to EcCDT-I (B) or PaCDT (C, D, and F). DNA content of CHO cells was monitored by flow cytometry as described in Materials and Methods. The effects of EcCDT-I (B) and PaCDT (C and D) on the CHO cell cycle (G_1 and G_2/M) were compared to the lysate of *E. coli* strain C600 (A) used as a negative control. The effect of rPaCDT (F) on CHO cell cycle (G_1 and G_2/M) was compared to that of *E. coli* strain BL21(DE3) carrying the empty vector pET-28a (F). *, $P < 0.05$ (Student *t* test, $n = 3$).

toxins. Although EcCDT-I showed cytotoxicity to Vero, HEp2, Int407, and Caco-2 cells, PaCDT was cytotoxic only to Vero and Caco-2 cells. Both toxins failed to show any cytotoxic effects on Y-1 and NIH/3T3 cells. To further examine whether the cytotoxic activity of PaCDT on CHO cells is specific, CHO cells were incubated with filter-sterilized lysate of *P. alcalifaciens* strains AH-31 or AS-1 in the presence of rabbit antiserum raised against rPaCdtB or filter-sterilized lysate of *E. coli* strain TAS-2 (rPaCDT) alone. CHO cell distention was not observed only when filter-sterilized lysate of *P. alcalifaciens* strain AH-31 or AS-1 was mixed with anti-rPaCdtB (Fig. 4H) but not with preimmunized rabbit serum (Fig. 4G). Furthermore, rPaCDT alone also caused morphological changes of CHO cells (Fig. 4F), which was similar to EcCDT-I, indicating that the CHO cell cytotoxic effect was most likely due to the CDT produced by *P. alcalifaciens* strain AH-31 or AS-1. We further explored the DNA contents of CHO cells treated with PaCDT. The filter-sterilized lysates of both parental *P. alcalifaciens* strains AH-31 (Fig. 5C) and AS-1 (Fig. 5D) caused G_2/M cell cycle arrest on CHO cells ($P < 0.05$) compared to that of the lysate of *E. coli* strain C600, a CDT-negative wild-type strain used as a control (Fig. 5A). Similarly, filter-sterilized lysate of *E. coli* strain TAS-2 producing rPaCDT (Fig. 5F) caused G_2/M cell cycle arrest on CHO cells ($P < 0.05$) compared to that of *E. coli* BL21(DE3) strain carrying the empty vector pET-28a (Fig. 5E).

Since EcCDT-I has been shown to cause phosphorylation of the histone H2AX, a sensitive marker for double-strand DNA breaks, we also examined whether PaCDT is involved in phosphorylation of the histone H2AX (γH2AX). As shown in Fig. 6, phosphorylated H2AX was visualized by direct immunofluorescence using antibodies against γH2AX, and a strong nuclear signal was detected in PaCDT-treated cells such as EcCDT-I-treated cells but not in control cells, indicating that PaCDT entered into the nucleus and induced DNA double-strand breaks of CHO cells.

Enterotoxic activity. Enterotoxicity of CDT produced by *P. alcalifaciens* was examined by suckling mouse assay. Crude rPaCDT prepared from *E. coli* strain TAS-2, rCT, or PBS was orally administered to each mouse, followed by an evaluation of the diarrheal score for each sample. rCT and PBS showed 100% positive and negative results, respectively. However, crude rPaCDT did not show any enterotoxicity (data not shown).

Subsequently, live bacteria were orally inoculated into suckling mice to see whether there was any fluid accumulation. Although 10^{10} CFU of *P. alcalifaciens* strain AS-1 did not cause any diarrhea in 12 mice tested, the same dose of *P. alcalifaciens* strain AH-31 could cause diarrhea in 7 of 12 mice. Further study is needed to prove the enterotoxic activity of purified PaCDT.

DISCUSSION

Despite tremendous efforts toward understanding the pathogenesis of *P. alcalifaciens*, it is still unclear how *P. alcalifaciens* causes diarrhea in humans. In the present study we show that *P. alcalifaciens* strains isolated from patients with diarrhea could produce CDT, and this is the first report, to our knowledge, regarding the

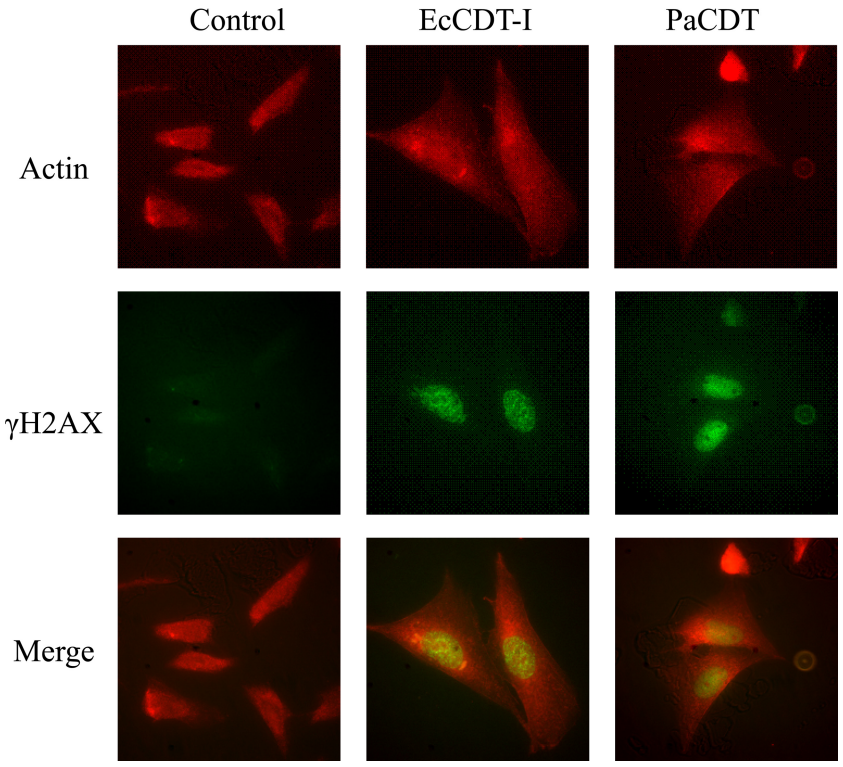

FIG 6 Genotoxic effect of PaCDT. CHO cells were treated with filter-sterilized lysates of bacteria producing EcCDT-I (GB1371) or PaCDT (AH-31). After 16 h of treatment, the cells were stained as described in Materials and Methods with Alexa Fluor 546-conjugated phalloidin (upper panels) or with fluorescein isothiocyanate-conjugated anti-phospho-histone H2AX (H2AX) monoclonal antibody (middle panels). EcCDT-I- and PaCDT-treated cells exhibited nuclear H2AX indicating host DNA double-strand breaks, enlarged nuclei and cell bodies, and the absence of mitotic features. Filter-sterilized lysate of *E. coli* C600 was used as a negative control. The experiment was repeated at least three times.

production of the toxin by the genus *Providencia*, including *P. alcalifaciens*.

Genus *Providencia*, belonging to the family *Enterobacteriaceae*, consists of five species: *P. alcalifaciens*, *P. stuartii*, *P. rettgeri*, *P. rustigianii*, and *P. heimbachae* (27). Among these, *P. alcalifaciens* has been described as a causative agent of diarrhea because a number of *P. alcalifaciens* strains were isolated from patients with diarrhea in developing countries (21, 22, 47, 48). Indeed, a case control study conducted by Albert et al. (3) demonstrated that *P. alcalifaciens* was associated with diarrhea in children in Bangladesh. Haynes and Hawkey (22) reported that *P. alcalifaciens* was associated with traveler's diarrhea. Yoh et al. (56) showed that not only *P. alcalifaciens* but also *P. rettgeri* in particular is an important pathogen for traveler's diarrhea. Furthermore, two large outbreaks of food poisoning caused by *P. alcalifaciens* have been reported from Japan and the Czech Republic (13, 36).

Several studies demonstrated that *P. alcalifaciens* is able to invade cultured epithelial cells (1, 21, 26). Invasion was also observed in intestinal tissues by using a removable intestinal tie adult rabbit diarrhea (RITARD) model and an adult rabbit ileal loop model (1, 35). Although invasion was considered as one of the virulence mechanisms to cause diarrhea by *P. alcalifaciens* strains, noninvasive *P. alcalifaciens* were also isolated from patients with diarrhea (21, 48). In our study, one strain, *P. alcalifaciens* AH-31, showed invasiveness to HeLa cells; however, another strain, *P. alcalifaciens* strain AS-1, did not show any invasiveness (data not shown). This observation suggests that invasiveness could be one of several possible virulence mechanisms. Therefore, other mechanisms by which *P. alcalifaciens* is involved in diarrhea have been considered but are not fully understood. Until the present study, no toxin was reported to be the virulence factor of *P. alcalifaciens*. CDT produced by *P. alcalifaciens* may be a candidate virulence factor in these strains other than invasiveness. Colony hybridization assay revealed that *cdt* genes are present only in certain strains of *P. alcalifaciens*. In addition to the cytotoxicity test, we also attempted to examine the enterotoxic activity of PaCDT in a suckling mouse assay. However, the suckling mouse assay did not show any enterotoxicity by crude concentrated rPaCDT, but one of the *P. alcalifaciens* strains (the strain AH-31) showed enterotoxicity when live bacteria were orally administered (Table 3). Based on these findings, it is not clear whether PaCDT is indeed a virulence factor for this pathogen, and further studies are needed to shed light on this aspect.

Some bacterial species, e.g., certain strains of *E. coli* carry *cdt*

TABLE 3 Suckling mouse assay with *Providencia alcalifaciens*

Sample	Diarrhea score (no. of positive animals/total no. of animals)[a]
E. coli C600	0 (0/12)
P. alcalifaciens AH-31	58 (7/12)
P. alcalifaciens AS-1	0 (0/12)
rCT[b]	100 (9/9)

[a] The animals excreting stained loose and/or watery feces over 24 h after sample administration were judged positive.
[b] Recombinant cholera toxin.

TABLE 4 Cytotoxicity of EcCDT-1 and PaCDT on various cell lines

Strain and sample	CD_{50}[a]							
	CHO	HeLa	Vero	HEp2	Int407	Caco-2	Y-1	NIH/3T3
E. coli								
GB1371 (EcCDT-I)	64 (70)	128 (140)	32 (35)	16 (18)	16 (18)	128 (140)	<1 (<1)	<1 (<1)
TAS-2 (rPaCDT)	8 (7.7)	<1 (<0.97)	1 (0.97)	<1 (<0.97)	<1 (<0.97)	4 (3.9)	<1 (<0.97)	<1 (<0.97)
P. alcalifaciens								
AH-31 (PaCDT)	2 (2.2)	<1 (<1.1)	<1 (<1.1)	<1 (<1.1)	<1 (<1.1)	1 (1.1)	<1 (<1.1)	<1 (<1.1)
AS-1 (PaCDT)	8 (12)	<1 (<1.5)	1 (1.5)	<1 (<1.5)	<1 (<1.5)	4 (6.0)	<1 (<1.5)	<1 (<1.5)

[a] The values indicate titers as the 50% cytotoxic dose (CD_{50}). Numbers in parentheses indicate the titer per mg of protein of the sample ($n = 3$).

genes, including its several variants (55), and other species, e.g., *C. jejuni* and *C. coli*, ubiquitously carry *cdt* genes in a species-specific manner (6). Although *cdt* genes in *C. jejuni* and *C. coli* are not associated with any mobile genetic element, CDT produced by *E. coli* has been demonstrated to be encoded on bacteriophage or plasmid (5, 43, 50, 51). In order to understand whether the *cdt* gene cluster is horizontally transferred among closely related species, the distribution of *cdt* genes was explored in *P. alcalifaciens*, as well as other *Providencia* spp., including *P. stuartii*, *P. rettgeri*, and *P. rustigianii*, and it was shown that only limited strains of *P. alcalifaciens* harbored the *cdt* genes. This result prompted us to further examine the possibility of horizontal transfer of Pa*cdt* genes. Initially, we attempted to isolate plasmid; however, no plasmid was detected in both *P. alcalifaciens* strains AH-31 and AS-1. Nucleotide sequence analysis of the flanking region of Pa*cdt* genes, however, revealed the presence of sequence predicted to encode a protein homologous to a transposase. The %GC content of each ORF or the entire region varied from 30 to 50% (Fig. 2 and Table 2), respectively. The %GC content of the genus *Providencia* has been reported to be ca. 39 to 42% (17). However, the %GC content of Pa*cdt* flanking regions showed mosaic structure of high and low %GC content, indicating that Pa*cdt* genes might have been acquired by horizontal gene transfer events, and later on it evolved further by repeated homologous recombination in various bacterial species or even in various bacterial genera.

A number of Gram-negative bacteria have been reported to produce CDT. CDT is a very unique bacterial protein toxin, which inhibits the cell cycle at G_2/M phase, leading to cell death and can enter into the nucleus and directly damage DNA. Thus, CDT is also called cyclomodulin or genotoxin. Comparative analysis of nucleotide and amino acid sequences of CDT revealed that CDT produced by *P. alcalifaciens* is closest to that produced by *S. boydii*. CDT thus far reported was generally active on CHO and HeLa cells (46, 51). However, PaCDT could cause cell distention and death of CHO, Vero, and Caco-2 cells, but it is not effective in other cells, including HeLa cells even when used at high concentration (Table 4). We cannot exclude the possibility that the toxin concentration was too low in our preparation to cause any cytotoxic effect to these cell lines. The receptor for EcCDT-I has been reported to be putative G protein-coupled receptor encoded by TMEM181 (11). These observations indicate that the receptor for PaCDT may be different from that of other CDTs, including EcCDT-I. Similar findings that CDTs produced from different bacteria, e.g., *A. actinomycetemcomitans*, *H. ducreyi*, *C. jejuni*, and *E. coli*, may display variable target cell tropism and may have different receptors as reported by Eshraghi et al. (16).

In conclusion, our data clearly demonstrated that *P. alcalifaciens* is a new member of CDT-producing bacterial family. However, in the present study, we were unable to demonstrate PaCDT as a possible virulence factor in *P. alcalifaciens*. Further investigation is necessary to determine the role of CDT in the pathogenesis of diarrhea caused by *P. alcalifaciens* and to clarify how the *cdt* genes was evolved and transferred among *P. alcalifaciens* strains.

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