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Campylobacter hyointestinalis Isolated from Pigs Produces Multiple Variants of Biologically Active Cytolethal Distending Toxin

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Campylobacter hyointestinalis **isolated from swine with proliferative enteritis often is considered to be pathogenic. While the precise virulence mechanisms of this species remain unclear, we have recently identified a cytolethal distending toxin (*cdt*) gene cluster in *C. hyointestinalis* isolated from a patient with diarrhea (W. Samosornsuk et al., J Med Microbiol, 27 July 2015, http://dx.doi.org/10.1099/jmm.0.000145). However, the sequences of the *cdt* genes in *C. hyointestinalis* were found to be significantly different and the gene products are immunologically distinct from those of other *Campylobacter* species. In this study, we demonstrate the presence of a second variant of the *cdt* gene cluster in *C. hyointestinalis*, designated *cdt-II*, while the former is named *cdt-I*. Sequencing of the *cdt-II* gene cluster and deduced amino acid sequences revealed that homologies between the subunits CdtA, CdtB, and CdtC of ChCDT-I and ChCDT-II are 25.0, 56.0, and 24.8%, respectively. Furthermore, the CdtB subunit of ChCDT-II was found to be immunologically unrelated to that of ChCDT-I by Ouchterlony double gel diffusion test. Recombinant ChCDT-II also induced cell distention and death of HeLa cells by blocking the cell cycle at G_2/M phase. Interestingly, the *cdt-II* genes were detected in all 23 animal isolates and in 1 human isolate of *C. hyointestinalis*, and 21 of these strains carried both *cdt-I* and *cdt-II* gene clusters. Altogether, our results indicate that ChCDT-II is an important virulence factor of *C. hyointestinalis* in animals.**

Campylobacter hyointestinalis was first isolated from swine with proliferative enteritis (1, 2). Subsequently, this organism was isolated from not only diseased but also healthy animals (3–6). Several recent reports indicate the isolation of increasing numbers of *C. hyointestinalis* organisms from patients with diarrhea (4, 7–12). Therefore, *C. hyointestinalis* is considered an emerging zoonotic pathogen of the genus *Campylobacter*.

Although several virulence factors and properties, such as attachment and invasion properties, various toxins, and type four and six secretion systems, etc., have been identified in *Campylobacter* spp. (13), the pathogenic mechanisms have not yet been fully understood. For *C. hyointestinalis*, there was almost no information on its virulence factor and mechanisms except for an uncharacterized cytotoxin reported by Ohya and Nakazawa (14). Although Pickett et al. reported that a part of the cytolethal distending toxin B subunit (*cdtB*) gene was amplified from *C. hyointestinalis* by PCR (15), it was not obvious that a biologically active cytolethal distending toxin (CDT) indeed is produced. Recently, Samosornsuk et al. have reported that *C. hyointestinalis* isolated from a diarrheal patient in Thailand contained a novel *cdt* gene cluster and produced a biologically active CDT, called ChCDT (16).

CDT holotoxin consists of three subunits, CdtA, CdtB, and CdtC, which are encoded by three physically linked genes, *cdtA*, *cdtB*, and *cdtC*, respectively (17). CdtA and CdtC are responsible for binding to a receptor molecule(s) present on the surface of susceptible mammalian cells, while CdtB, having DNase I-like activity, is responsible for toxin activity. Asakura et al. have reported that *cdt* genes are ubiquitously present in *C. jejuni*, *C. coli*, and *C. fetus* and are highly homologous within species but less homologous among species (18; N. Hatanaka, unpublished). Based on these findings, a *cdt* gene-based multiplex PCR assay for the detection of *C. jejuni*, *C. coli*, and *C. fetus* has been developed (19) and utilized for the identification of these species from poultry and diarrheal patients (20, 21). Furthermore, Kamei et al. developed a *cdtB* gene-based PCR-restriction fragment length polymorphism (RFLP) assay for the detection and differentiation of 7 *Campylobacter* species, i.e., *C. jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. lari*, *C. helveticus*, and *C. upsaliensis*, and successfully identified the species of 132 *Campylobacter* strains, failing to identify only 3 *C. hyointestinalis* strains (22). However, it is unclear whether these *C. hyointestinalis* strains contain *cdt* genes or not. The CDT activity produced by sonic lysate of *C. hyointestinalis* strain Ch022, isolated from a patient with diarrhea, was not neutralized by antiserum against recombinant CdtB of *C. hyointestinalis* strain Ch022 (16), suggesting the production of an unknown toxin(s) by the *C. hyointestinalis* strain Ch022. This also could be the case for *C. hyointestinalis* strains in which the *cdtB* gene was not amplifiable by the PCR-RFLP assay (22).

In this study, we investigated whether the *cdt* gene-negative *C. hyointestinalis* strains isolated from pig, pork, and cow harbor *cdt* variant genes and produce a biologically active CDT. Such analysis allowed us to detect successfully a novel *cdt* gene vari-

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TABLE 1 CDT activity and distribution of *cdt* genes in bacterial strains

Bacterium	Strain	Origin	CDT titer[a]	Colony hybridization[g] (A/B/C)	
				cdt-I[b]	*cdt-II*[c]
C. hyointestinalis	Ch022	Human[f]	128	+/+/+	+/+/+
	ATCC 35217^T	Pig[f]	2,048	−/−/−	+/+/+
	SS	Pork	1,024	−/−/−	+/+/+
	1-1	Pig[f]	256	+/+/+	+/+/+
	10-1	Gorilla	512	+/+/+	+/+/+
	87-4	Monkey	64	+/+/+	+/+/+
	84-6	Elephant	256	+/+/+	+/+/+
	2003	Cow	32	+/+/+	+/+/+
	2030	Cow	128	+/+/+	+/+/+
	2032	Cow	32	+/+/+	+/+/+
	2033	Cow	1,024	+/+/+	+/+/+
	2034	Cow	512	+/+/+	+/+/+
	2035	Cow	128	+/+/+	+/+/+
	2037	Cow	128	+/+/+	+/+/+
	2038	Cow	2,048	−/−/−	+/+/+
	2039	Cow	4	+/+/+	+/+/+
	2073	Cow	128	+/+/+	+/+/+
	3014	Cow	64	+/+/+	+/+/+
	3158	Cow	512	+/+/+	+/+/+
	3197	Cow	64	+/+/+	+/+/+
	3477	Cow	128	+/+/+	+/+/+
	3535	Cow	64	+/+/+	+/+/+
	3839	Cow	128	+/+/+	+/+/+
	3857	Cow	32	+/+/+	+/+/+
E. coli	C600	NA	ND	−/−/−	−/−/−
C. jejuni[d]	81-176	Human	256	ND/−/ND	ND/−/ND
C. coli[d]	ATCC 33559^T	Pig	ND	ND/−/ND	ND/−/ND
C. fetus[d]	ATCC 27374^T	Sheep	ND	ND/−/ND	ND/−/ND
C. lari[d]	ATCC 43675	Human	ND	ND/−/ND	ND/−/ND
C. upsaliensis[d]	ATCC 43954^T	Dog	ND	ND/−/ND	ND/−/ND
C. helveticus[d]	ATCC 51209^T	Cat	ND	ND/−/ND	ND/−/ND
C. concisus[e]	ATCC 33237^T	Human	ND	ND/−/ND	ND/−/ND
C. curvus[e]	ATCC 35224^T	Human	ND	ND/−/ND	ND/−/ND
C. hominis[e]	ATCC BAA-381^T	Human	ND	ND/−/ND	ND/−/ND
C. mucosalis[e]	ATCC 49352	Pig	ND	ND/−/ND	ND/−/ND

[a] Reciprocal of the highest dilution that resulted in cytoplasmic distention in >50% of cells.
[b] Specific probe was prepared from *C. hyointestinalis* strain Ch022.
[c] Specific probe was prepared from *C. hyointestinalis* strain ATCC 35217^T.
[d] Presence of *cdt* genes has been reported.
[e] Presence of *cdt* genes has not been reported.
[f] Isolated from proliferative enteritis infection.
[g] NA, not applicable; ND, not done; +, detected; −, not detected.

ant not only in three *C. hyointestinalis* strains which were *cdt* negative by the PCR-RFLP assay (22) but also in all other *C. hyointestinalis* strains examined in this study. To the best of our knowledge, this is the first report showing that *C. hyointestinalis* strains isolated from animals produce CDT; indeed, some of the strains produced two different variants of the toxin, which are biologically active.

(This study was performed in partial fulfillment of the requirements for the completion of the Ph.D. thesis of K. Kamei from the Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. *C. jejuni* strain K328 (23) was used as a negative control for the cytotoxicity assay. *Escherichia coli* strains JM109 and BL21(DE3) were used for cloning and expression of recombinant proteins, respectively. *Campylobacter* strains except *C. concisus*, *C. curvus*, *C. hominis*, and *C. mucosalis* were grown on blood agar (blood agar base no. 2 [Oxoid Ltd., Basingstoke, United Kingdom] supplemented with 5% [vol/vol] defibrinated horse blood [Nippon Bio-Supp. Center, Tokyo, Japan]) at 37°C for 2 days or more under microaerobic conditions (5% O_2, 7.5% CO_2, 7.5% H_2, 80% N_2). *C. concisus*, *C. curvus*, and *C. hominis* were grown on blood agar containing 6% formate and fumarate at 37°C for 2 days or more under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). *C. mucosalis* was grown on blood agar at 37°C for 2 days or more under anaerobic conditions. *E. coli* strains were grown in Luria-Bertani (LB; Becton, Dickinson and Company, Franklin Lakes, NJ) broth at 37°C overnight. *E. coli* strains carrying recombinant plasmids were grown overnight in LB broth supplemented with 50 μg/ml ampicillin or 30 μg/ml kanamycin at 37°C with shaking.

DNA preparation. Genomic DNA of *C. hyointestinalis* was purified by using an Isoplant DNA extraction kit as directed by the manufacturer (Nippon Gene Co., Ltd., Tokyo, Japan).

PCR and preparation of DNA probe for hybridization assay. The target genes were amplified from *C. hyointestinalis* strain Ch022 or ATCC 35217^T by PCR using the respective primer sets listed in Table S1 in the supplemental material. All PCR mixtures contained 0.5 μM (each) primers, 0.1 to 1.0 ng of genomic DNA, 0.2 mM deoxynucleoside triphosphate (dNTP) mixture, 1× *Ex Taq* DNA polymerase buffer, and 1.0 U of *Ex Taq* DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) in a 40-μl reaction volume. PCR was performed in a TaKaRa PCR thermal cycler (TaKaRa Bio Inc.) or Applied Biosystems GeneAmp PCR 9700 (Life Technologies Co., Carlsbad, CA). PCR products were analyzed by 1 to 2% agarose gel electrophoresis, and bands were visualized with UV light after staining with ethidium bromide (1 μg/ml). Images were captured on a Bio-Rad Chemi Doc system (Bio-Rad Laboratories, Inc., Hercules, CA). A PCR-amplified target gene was purified by using a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany), labeled with [α-^{32}P]dCTP (111 TBq mmol^{-1}) by a random priming method using a Multiprime DNA labeling system (GE Healthcare UK Ltd., Buckinghamshire, England), and used as a gene probe for hybridization assay.

Colony hybridization. Distribution of the *cdt* gene cluster among different isolates was examined by colony hybridization assay as described previously, with minor modifications (24). In brief, strains were grown on a nitrocellulose membrane (Schleicher and Schuell BioScience GmbH, Dassel, Germany) overlaid on appropriate agar plates with proper culture conditions. Colonies then were lysed and DNA was denatured *in situ* by the alkaline lysis method followed by UV cross-linking with a UV cross-linker (CX-2000; UVP LLC, Upland, CA). The processed nitrocellulose membranes were hybridized with the target gene probes under suitable buffer conditions, and radioactivity was visualized by an FLA-7000 (GE Healthcare UK Ltd.).

Preparation of bacterial sonic lysate. Bacterial strains were cultured as described above, harvested, and suspended in sterile phosphate-buffered saline (PBS) at pH 7.4. The optical density at 600 nm was adjusted to 10, and the suspension was sonicated 2 or 3 times for a 1-min duration and stored for 1 min on ice using a handy sonicator (UR-20P; Tomy Seiko Co., Ltd., Tokyo, Japan). Bacterial sonic lysate was centrifuged at 12,000 × *g* for 10 min, and supernatant was collected, filtered through a 0.22-μm-pore-size filter membrane (Asahi Glass Co., Ltd., Tokyo, Japan), diluted with sterile PBS, and used for the cytotoxicity assay.

Cytotoxicity assay. HeLa cells were cultured in minimum essential medium (MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Life Technologies Co.), GlutaMAX (Life Technologies Co.), and an antibiotic cocktail of streptomycin and penicillin (Life Technologies Co.). About 5.0 × 10^3 cells in 100 μl of culture medium were seeded in each well of a 96-well plate (Corning, NY) and incubated with 10 μl of filter-sterilized bacterial lysates at 37°C under 5% CO_2 in air. After 24 to 120 h of incubation, the cells were stained with Giemsa solution by using Diff-quick (Sysmex Co., Hyogo, Japan), and cytotoxic effects were observed under a Leica DM16000 B microscope (Leica Microsystems, Mannheim, Germany). *C. hyointestinalis* strain Ch022 (21) and *C. jejuni* strain K328 (23) were used as a positive- and a negative-control strain, respectively. The CDT titer was determined as the reciprocal of the highest dilution that resulted in cytoplasmic distention in >50% of cells.

Analysis of cell cycle inhibition. About 2.5 × 10^5 cells in 4 ml of culture medium were seeded in a 25-cm^2 flask (Corning) and incubated with 100 μl of filter-sterilized bacterial lysate at 37°C under 5% CO_2 in air. After 24 h of incubation, the cells were collected and fixed with 70% ethanol on ice for 1 h. The cells were stained with propidium iodide (PI; 50 μg/ml) in PBS containing 0.25 mg/ml of RNase A (Sigma-Aldrich, St. Louis, MO) at 4°C for 30 min under dark conditions. Flow cytometry analysis of DNA content of the cells was performed with a FACSCalibur flow cytometer (Becton, Dickinson and Company), and 10,000 events were collected. Cell cycle analysis was performed by Cell Quest Pro software (Becton, Dickinson and Company).

Fluorescence microscopy. About 1.0 × 10^4 cells in 200 μl of culture medium were seeded in each well of a glass slide (Nalge Nunc International, Rochester, NY) and allowed to adhere for 24 h. Cells were incubated with 10 μl of filter-sterilized bacterial lysates at 37°C for 16 h under 5% CO_2 in air. Cells were fixed with 3.7% formaldehyde for 10 min, followed by treatment with 0.5% Triton X-100 for 20 min and 1.0% bovine serum albumin (BSA)–PBS for 30 min. γ-H2AX in HeLa cells was stained with anti-phospho-histone-H2AX (Ser139) polyclonal antibody (Enzo Life Sciences, Inc., Farmingdale, NY) at 37°C for 30 min and Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies Co.) at 37°C for 1 h. Intracellular F-actin was stained with Alexa Fluor 546-conjugated phalloidin (Life Technologies Co.) at 37°C for 1 h. Fluorescence was observed under an epifluorescent Leica DM2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Cloning. The PCR product was purified by using a QIAquick gel extraction kit and ligated into a pT7Blue vector (Merck KGaA, Darmstadt, Germany) or a pSTBlue-1 vector (Merck KGaA). The ligation mixture was transformed into *E. coli* strain JM109. A recombinant clone harboring insert DNA was selected on LB agar containing 50 μg/ml ampicillin supplemented with 50 μl each of 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The white colony was selected, and recombinant plasmid DNA was isolated from *E. coli* by the alkaline lysis method.

DNA sequencing. The sequencing reaction was performed by the chain termination method with the BigDye Terminator v1.1 cycle sequencing kit (Life Technologies Co.). Nucleotide sequences were determined using an ABI PRISM 3100-Avant genetic analyzer (Life Technologies Co.). Nucleotide sequences obtained in this study were analyzed using the DNA Lasergene software package (DNASTAR Inc., Madison, WI). Homology searches were performed using BLAST and FASTA programs, made available by the National Center for Biotechnology Information (NCBI). Nucleotide sequence and amino acid sequence alignments were analyzed by ClustalW of MegAlign (DNASTAR Inc.).

Southern blotting. The genomic DNA (400 ng) of *C. hyointestinalis* was digested with DraI (New England Biolabs Inc., Ipswich, MA), followed by electrophoresis using 1% SeaKem LE agarose gel (Lonza, Basel, Switzerland) and transfer of DNA to a nylon membrane (PerkinElmer Life Sciences, Inc., Boston, MA). The DNA probe was prepared from the PCR product amplified by degenerative primers (15, 25), and hybridization was carried out as described above. The *cdtB* gene-specific probe was prepared from *C. hyointestinalis* strain Ch022 and used as a probe.

Genome walking. The random primers, target primers, and sequence primers are summarized in Table S2 in the supplemental material. Genome walking was performed to determine the nucleotide sequence of the *cdt* gene cluster and its flanking region as described previously, with slight modifications (18). In brief, 50 ng of genomic DNA was randomly extended for only 1 cycle of 5 min at 94°C, 30 s at 30°C, and 30 s at 72°C, with 0.4 μM random primer, 0.2 mM dNTP mixture, 1× *Ex Taq* DNA polymerase buffer, and 2.5 U of *Ex Taq* DNA polymerase in a 25-μl reaction volume. Further, PCR amplification was performed with 0.4 μM target primer, 0.2 mM dNTP mixture, 1× *Ex Taq* DNA polymerase buffer, and 7.5 U of *Ex Taq* DNA polymerase in a 100-μl reaction volume. The cycling parameters were 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 35 cycles, with a final extension time of 5 min. The amplified fragments were sequenced with primers listed in Table S2 in the supplemental material.

Expression and preparation of recombinant protein. The *cdt-IIA*, *cdt-IIB*, and *cdt-IIC* genes (open reading frames [ORFs] 11 to 13 in Fig. 2; also see Table S3 in the supplemental material) were PCR amplified using the genomic DNA of *C. hyointestinalis* strain ATCC 35217^T as the template (see Table S1). Each of these amplified DNA fragments was digested with either EcoRI and HindIII or EcoRI and SacI (New England Biolabs Inc.) and cloned into the region downstream of the EcoRI site of the modified pET28a+ vector (Novagen, New Canaan, CT). This vector encoded His_6 sequence and a tobacco etch virus protease cleavage (TEV)

sequence upstream of the EcoRI restriction site. The recombinant plasmid was transformed into *E. coli* strain BL21(DE3), and a transformant was selected and grown in LB broth containing 30 μg/ml kanamycin at 37°C. When the optical density at 600 nm of the culture reached 0.3 to 0.6, IPTG was added to a final concentration of 0.1 to 1.0 mM, and the culture was further incubated at 37°C for 3 h with vigorous shaking. *E. coli* cells were harvested by centrifugation at 6,000 × *g* at 4°C for 15 min, suspended in PBS, sonicated on ice using an Astrason ultrasonic processor (Heat System-Ultrasonics Inc., Farmingdale, NY), and centrifuged again at 48,000 × *g* for 20 min at 4°C. Recombinant ChCdt-IIB (rChCdt-IIB) was purified from the soluble fraction by using a Ni-Sepharose column (GE Healthcare UK, Ltd.) and eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 200 mM imidazole. The purity of rChCdt-IIB was confirmed by SDS–15% PAGE. The concentration of protein was measured by Bradford assay (Bio-Rad Laboratories, Inc.). In the case of rChCdt-IIA and rChCdt-IIC, the insoluble fraction was suspended with sterile PBS and applied to SDS–15% PAGE. Bands of approximately 25 and 28 kDa in size, which correspond to the sizes of ChCdt-IIA and ChCdt-IIC, respectively, were cut out from polyacrylamide gels and used for immunization.

Preparation of antisera against rChCdt-II subunits. Antiserum against rChCdt-IIA, rChCdt-IIB, or rChCdt-IIC was generated by immunizing an 8-week-old male New Zealand White rabbit (Oriental Yeast Co., Ltd., Tokyo, Japan) with His-tagged fusion protein of subunit A, B, or C, respectively, of ChCDT-II toxin. Briefly, 370 μg of purified rChCdt-IIB or a polyacrylamide gel slice containing rChCdt-IIA or rChCdt-IIC (approximately 200 μg) was injected into four sites, i.e., subcutaneously into the shoulders and intramuscularly into the thighs, every 2 weeks first with Freund complete adjuvant (Becton, Dickinson and Company) and subsequently with Freund incomplete adjuvant (Becton, Dickinson and Company) for 12 to 14 weeks. After the last immunization, the rabbit was boosted intravenously with 100 μg of purified rChCdt-IIB or intramuscularly with a polyacrylamide gel slice containing subunit proteins rChCdt-IIA and rChCdt-IIC with Freund incomplete adjuvant. After 3 to 5 days, the immunized rabbit was anesthetized with ketamine (35 mg/kg of body weight) and xylazine (5 mg/kg), blood was collected, and serum was obtained by centrifugation at 1,500 × *g* for 10 min. The polyclonal antibody against each subunit was purified by using an rProtein A Sepharose fast flow (GE Healthcare UK, Ltd.) and eluted with 20 mM glycine-HCl containing 150 mM NaCl (pH 3.0). All animal experiments were performed according to the Guidelines for Animal Experimentation of Osaka Prefecture University and approved by the Animal Experiment Committee of Osaka Prefecture University.

Ouchterlony gel diffusion test. rChCdt-IB and antiserum against rChCdt-IB were prepared as described previously (16). The double gel diffusion test was carried out with 1.2% Noble agar (Becton, Dickinson and Company) in 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. Fifteen microliters of antiserum or 500 ng of recombinant protein was placed in a well. The plate was placed in a humidified chamber at room temperature for about 24 h. The plate was stained with 0.5% Coomassie brilliant blue (CBB) dissolved in a solution of 50% methanol and 10% acetic acid in distilled water and destained with the same solution without CBB.

Expression of rChCDT-II and cytotoxicity assay. The *cdt-IIABC* genes (ORFs 11 to 13 in Fig. 2; also see Table S3 in the supplemental material) were PCR amplified using the genomic DNA of *C. hyointestinalis* strain ATCC 35217^T as the template (see Table S1). PCR-amplified DNA fragments were digested with BsaI and BamHI (New England Biolabs Inc.) and cloned upstream of the BamHI site of the modified pET28a+ vector. This vector, termed the TH-pET vector, encoded a TEV sequence, His_6 sequence, and stop codon downstream of the BamHI restriction site. The recombinant plasmid was transformed into the *E. coli* strain BL21(DE3) and a recombinant clone was selected, followed by preparation of its sonic lysate and use for cytotoxicity assay as well as analysis of cell cycle inhibition as described before. The TH-pET vector without *cdt-IIABC* genes was used as a negative control.

Neutralization assay with anti-CDT antibodies. Filter-sterilized bacterial lysates were diluted with sterile PBS (for *C. hyointestinalis* strains ATCC 32517^T and Ch022, 2,048- and 64-fold dilutions, respectively, were prepared; for *C. jejuni* strain 81-176, a 128-fold dilution was used; for the *E. coli* strain carrying the TH-pET vector with *cdt-IIABC* genes, a 16-fold dilution was made). Each sample was mixed with sterile PBS, anti-rChCdt-IIA, or anti-rChCdt-IIC antibody (1 mg/ml) in equal amounts of toxin preparation and preincubated at 37°C for 1 h. About 5.0 × 10^3 HeLa cells in 100 μl of culture medium were seeded in each well of a 96-well plate, followed by incubation with 10 μl of sample at 37°C under 5% CO_2 in air. After 72 h of incubation, cells were stained with Giemsa solution by using Diff-quick as described above.

Western blotting. Filter-sterilized sonic lysates of each *C. hyointestinalis* strain and serially diluted rChCdt-IIA protein (0.32 to 5.0 ng) were analyzed by 4 to 15% gradient SDS-PAGE (Bio-Rad) and blotted onto a polyvinylidene difluoride (PVDF) membrane (pore size, 0.45 μm; Merck KGaA) by using a semidry transfer cell (Trans Blot SD, Bio-Rad). The membrane then was blocked with PBS containing 4% ECL prime blocking reagent (GE Healthcare UK, Ltd.) at room temperature for 2 h, followed by treatment with 5,000-times-diluted anti-rChCdt-IIA serum containing 10 ng/ml each of rChCdt-IA and rChCdt-IIC proteins. After incubation at room temperature for 2 h, the membrane was washed with PBS-T (0.01% Tween 20 in PBS) and incubated with 20,000-times-diluted horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (GE Healthcare UK, Ltd.) at room temperature for 2 h, followed by washing with PBS-T and detection of reactive protein by using an Amersham ECL prime kit (GE Healthcare UK, Ltd.).

Nucleotide sequence accession number. The nucleotide sequence of the *cdt-II* gene and its flanking region has been registered in the DNA Data Bank of Japan (DDBJ) under accession no. AB373951.

RESULTS

Genotoxic activity of *C. hyointestinalis* strains ATCC 35217^T, 2038, and SS. To see if *C. hyointestinalis* strains ATCC 35217^T, 2038, and SS, which were identified as *cdt* gene negative by the PCR-RFLP assay (22), produce any cytotoxic activity, filter-sterilized lysates of these *C. hyointestinalis* strains were prepared and used for the cytotoxicity assay on HeLa cells. In this assay, *C. hyointestinalis* strain Ch022 was used as a positive control. After 48 h of incubation, distention of HeLa cells was observed in all strains tested with the preparations (Fig. 1A to D), and further incubation up to 120 h resulted in the formation of multinuclei and cell death (data not shown). The filter-sterilized lysate of *C. jejuni* strain K328, used as a negative control, did not show any morphological changes in HeLa cells (Fig. 1E). The CDT titer was determined as the reciprocal of the highest dilution that resulted in cytoplasmic distention in >50% of cells, and the titers are summarized in Table 1. Interestingly, the titers of *C. hyointestinalis* strains ATCC 35217^T, 2038, and SS were higher than those of *C. hyointestinalis* strains that were demonstrated to possess *cdt* genes (22). We further explored the DNA contents of HeLa cells after 24 h of cultivation. The filter-sterilized lysates of *C. hyointestinalis* strains ATCC 35217^T, 2038, SS, and Ch022 caused G_2/M cell cycle arrest, but that of *C. jejuni* strain K328 did not show any such defect (see Fig. S1 in the supplemental material).

We further evaluated whether the filter-sterilized lysates of these three *C. hyointestinalis* strains (ATCC 35217^T, 2038, and SS) could cause the phosphorylation of histone H2AX. For this purpose, phosphorylated H2AX was visualized by direct immunofluorescence using antibodies against γ-H2AX, and a strong nuclear signal was detected in HeLa cells when treated with sonic lysate of

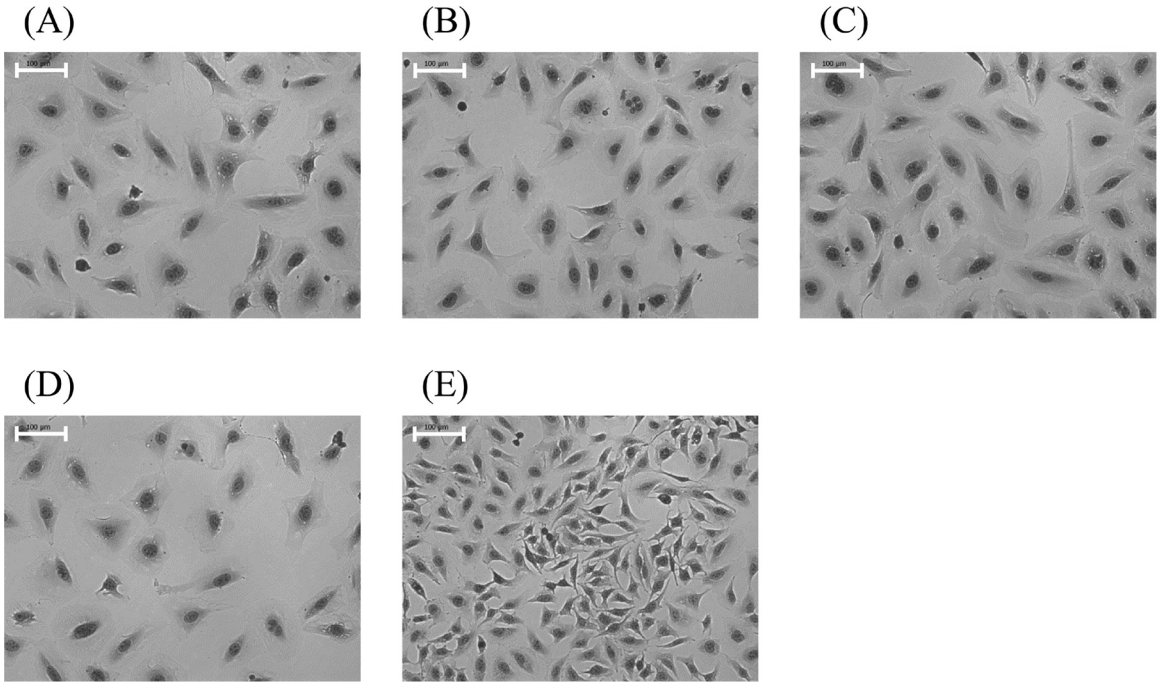

FIG 1 Cytotoxicity of the filter-sterilized lysates of *C. hyointestinalis*. HeLa cells were incubated for 48 h with filter-sterilized lysate of *C. hyointestinalis* strains ATCC 35217^T (A), SS (B), 2038 (C), and Ch022 (D) and *C. jejuni* strain K328 (E). Scale bars correspond to 100 μm. Magnification, ×100.

C. hyointestinalis strains ATCC 35217^T, 2038, and SS (see Fig. S2 in the supplemental material). These data indicated that *C. hyointestinalis* strains ATCC 35217^T, 2038, and SS produce CDT(s) and possess the *cdt*-like gene(s), which may be different from that identified in *C. hyointestinalis* strain Ch022 (16).

Distribution of the *cdt* gene cluster (*cdt-I*) in *C. hyointestinalis*. Since the *cdtB* gene was not amplified from the 3 *C. hyointestinalis* strains ATCC 35217^T, 2038, and SS by the PCR-RFLP assay as described previously (22), we conducted a colony hybridization assay using the *cdtA*, *cdtB*, and *cdtC* gene probes prepared from *C. hyointestinalis* strain Ch022 (20) to see if the *cdt*-like gene(s) is present in these *C. hyointestinalis* strains. In addition to these three strains, we tested 20 more *C. hyointestinalis* strains, isolated from various animals, which were positive for the *cdtB* gene by the PCR-RFLP assay (22).

All probes hybridized with the 20 strains examined and the *C. hyointestinalis* strain Ch022, which was used as a positive control (Table 1). As expected, *C. hyointestinalis* strains ATCC 35217^T, 2038, and SS and *E. coli* strain C600, used as a negative control, did not hybridize with any of the probes used, confirming that the *cdt* gene cluster might not be ubiquitously present in the case of *C. hyointestinalis*.

Identification of a partial *cdt*-like gene from *C. hyointestinalis* strain ATCC 35217^T. *C. hyointestinalis* strain ATCC 35217^T, isolated from swine with proliferative enteritis (1, 2), was selected for further analysis of *cdt* genes and their gene products. To detect the *cdt*-like genes in this type strain, PCR was carried out with degenerative primers designed from the conserved regions of the *cdt* genes found among *C. jejuni*, *C. coli*, and *E. coli* strains as described previously (15, 25). A band of the expected size of 960 bp indeed was PCR amplified from *C. hyointestinalis* strain ATCC 35217^T using the *cdtA*-based (GNW) and *cdtB*-based (WMI1) degenerative primers (see Fig. S3A in the supplemental material). The 960-bp PCR product was purified and cloned into the pT7Blue vector, followed by sequencing of the insert DNA. Sequence analysis of the insert DNA revealed homology with the *cdt* gene sequences available in the databank, and the sequence also showed 55.7% homology to the corresponding region of the *cdt* gene of *C. hyointestinalis* strain Ch022 (data not shown).

A Southern hybridization assay was carried out using the DraI-digested genomic DNA of *C. hyointestinalis* strains ATCC 35217^T and Ch022, and as a probe, a 960-bp PCR-amplified fragment (see Fig. S3A in the supplemental material) of *C. hyointestinalis* strain ATCC 35217^T or the *cdtB* gene-specific probe prepared from *C. hyointestinalis* strain Ch022 was used. The 960-bp DNA probe hybridized with fragments of DraI-digested genomic DNA of *C. hyointestinalis* of two different sizes (~750 and ~1,500 bp) (see Fig. S3B, lanes 1 and 2). On the other hand, the *cdtB* gene-specific probe hybridized with a fragment of ~2,500 bp of DraI-digested genomic DNA of *C. hyointestinalis* strain Ch022 but not with that of *C. hyointestinalis* strain ATCC 35217^T (see Fig. S3B, lanes 3 and 4). These data indicated that the PCR product amplified by degenerative primers from *C. hyointestinalis* strain ATCC 35217^T was a variant of a previously identified *cdt* gene of *C. hyointestinalis* strain Ch022. However, it should be noted that the *cdt* genes found in *C. hyointestinalis* strain ATCC 35217^T also were present in *C. hyointestinalis* strain Ch022 (see Fig. S3B, lanes 1 and 2).

Cloning and characterization of the newly identified *cdt* genes (*cdt-II*). Since *C. hyointestinalis* strain Ch022 harbors the *cdt* variant genes and we have identified in this study the presence of another variant of *cdt* genes in the ATCC type strain of *C. hyointestinalis*, to avoid confusion we named the former gene cluster *cdt-I* and the latter one *cdt-II*. To understand the gene cluster

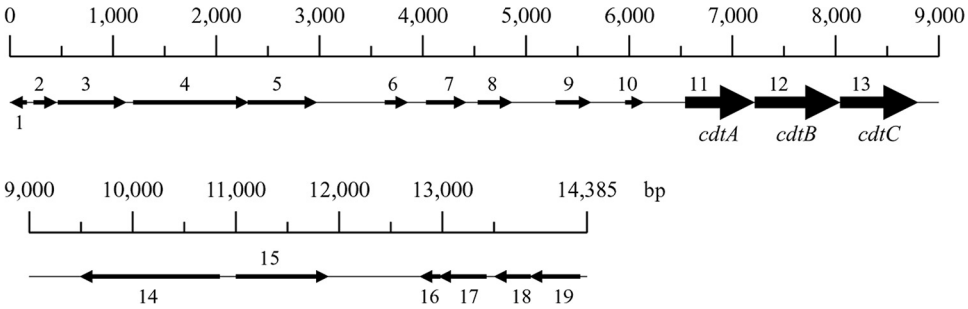

FIG 2 Schematic representation of the *cdt-II* genes and flanking regions of the *C. hyointestinalis* strain ATCC 35217^T. Thick arrows indicate *cdt-II* genes (*cdtABC*), and thin arrows indicate ORFs present in the flanking regions.

further, the entire nucleotide sequence of *cdt-II* in *C. hyointestinalis* strain ATCC 35217^T was determined by the genome walking method. A 14,385-bp fragment was sequenced, and the results are summarized in Fig. 2 (also see Table S3 in the supplemental material). Such analysis identified in the genome of *C. hyointestinalis* strain ATCC 35217^T the presence of 19 open reading frames (ORFs), including three continuous ORFs (ORFs 11 to 13) which are homologous to CdtA, CdtB, and CdtC of *C. hyointestinalis* strain Ch022 and those found in other *Campylobacter* spp. (see Tables S3 and S4). These *cdtA*-, *cdtB*-, and *cdtC*-like genes are 675, 825, and 753 bp in size, respectively, and they code for proteins of predicted molecular masses of 24.9, 30.2, and 28.2 kDa, respectively. The putative amino acid residues (corresponding to ChCdt-IB E61, E87, R114, R139, H155, G186-N190, D225, and S257-V262), important for DNase I activity, are conserved in ORF 12 except for two amino acids (see details in Fig. S2B) (17). Interestingly, homologies between the deduced amino acid sequences of the CdtA, CdtB, and CdtC mature proteins of two *C. hyointestinalis* strains, Ch022 and ATCC 35217^T, were only 25.0, 56.0, and 24.8%, respectively. The ranges of amino acid sequence homology between ChCDT-II and CDTs of other campylobacters (*C. jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. lari*, and *C. upsaliensis*) are 22.8 to 33.6% for CdtA, 52.6 to 56.6% for CdtB, and 24.8 to 39.5% for CdtC (see Table S4). Furthermore, the putative genes related to the prophage also were found to be present in the upstream region of *cdt-II* (ORFs 6 to 10 in Fig. 2 and Table S3).

Immunological relationship between rChCdt-IB and rChCdt-IIB. Since the deduced amino acid sequence homology between ChCDT-I and ChCDT-II is low (see Table S4 in the supplemental material), the immunological relationship between ChCdt-IB and ChCdt-IIB was examined by the Ouchterlony double gel diffusion test. As shown in Fig. 3, although rChCdt-IB and rChCdt-IIB were reactive to antiserum against rChCdt-IB and rChCdt-IIB, respectively, rChCdt-IB and rChCdt-IIB were not reactive to antiserum against rChCdt-IIB and rChCdt-IB, respectively. These data indicate that ChCdt-IB and ChCdt-IIB are immunologically unrelated.

Genotoxic activity of rChCDT-II. To evaluate the biological activity of ChCDT-II, the *cdt-II* gene cluster (ORFs 11 to 13 in Fig. 2; also see Table S3 in the supplemental material) was cloned and expressed in *E. coli* strain BL21(DE3). The filter-sterilized lysate of recombinant *E. coli* (*cdt-II*$^+$) induced cell distension, giant nuclei, multinuclei, and death of HeLa cells (see Fig. S5A). *E. coli* strain BL21(DE3) carrying the empty vector TH-pET was used as a negative control, and lysate of these cells did not show any morphological changes in HeLa cells. Furthermore, the filter-sterilized lysate of recombinant *E. coli* (*cdt-II*$^+$) caused G_2/M cell cycle arrest, but this activity was not found when the lysate of *E. coli* cells carrying the empty vector TH-pET was used (see Fig. S5B).

We further evaluated whether the CDT activity of *C. hyointestinalis* wild-type strains was due to ChCDT-II. As described above, the sonic lysate of recombinant *E. coli* (*cdt-II*$^+$) and *C. hyointestinalis* strains ATCC 35217^T and Ch022 induced HeLa cell distention (Fig. 4D, J, and M). The distending activities of these cell lysates were completely neutralized by antibody against either rChCdt-IIA (Fig. 4E, K, and N) or rChCdt-IIC (Fig. 4F, L, and O). On the other hand, the distending activity of the sonic lysate of *C. jejuni* strain 81-176 was not neutralizable by the antibody against rChCdt-IIA or rChCdt-IIC (Fig. 4G to I). Western blotting revealed that anti-rChCdt-IIA serum was reactive to a band of the expected size (25 kDa) of sonic lysates prepared from *C. hyointestinalis* strains ATCC 35217^T and Ch022 (data not shown). This antiserum did not react with any protein in the sonic lysate of *C. jejuni* strain 81-176 (data not shown). These data indicated that *C. hyointestinalis* strains ATCC 35217^T and Ch022 produced ChCDT-II, and the cytotoxicities of these strains against HeLa cells most probably were due to ChCDT-II. Furthermore, the concentration of ChCdt-IIA in sonic lysate of *C. hyointestinalis* strain ATCC 35217^T was semiquantified by Western blotting, and it was estimated to be about 400 pg/μl (corresponding to 1.3 ng/μl of ChCDT-II). Since the CDT titer of this sonic lysate was 2,048 (Table 1), the 50% cytotoxic dose (CD_{50}) of ChCDT-II was found to be about 59 pg/ml.

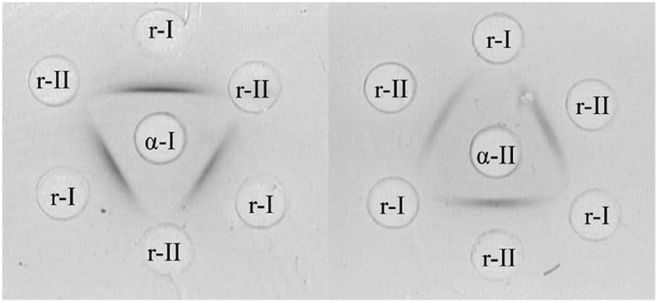

FIG 3 Immunological relationship between rChCdt-IB and rChCdt-IIB. Ouchterlony double gel diffusion tests were carried out with purified rChCdt-IB (r-I), purified rChCdt-IIB (r-II), anti-rChCdt-IB serum (α-I), and anti-rChCdt-IIB serum (α-II).

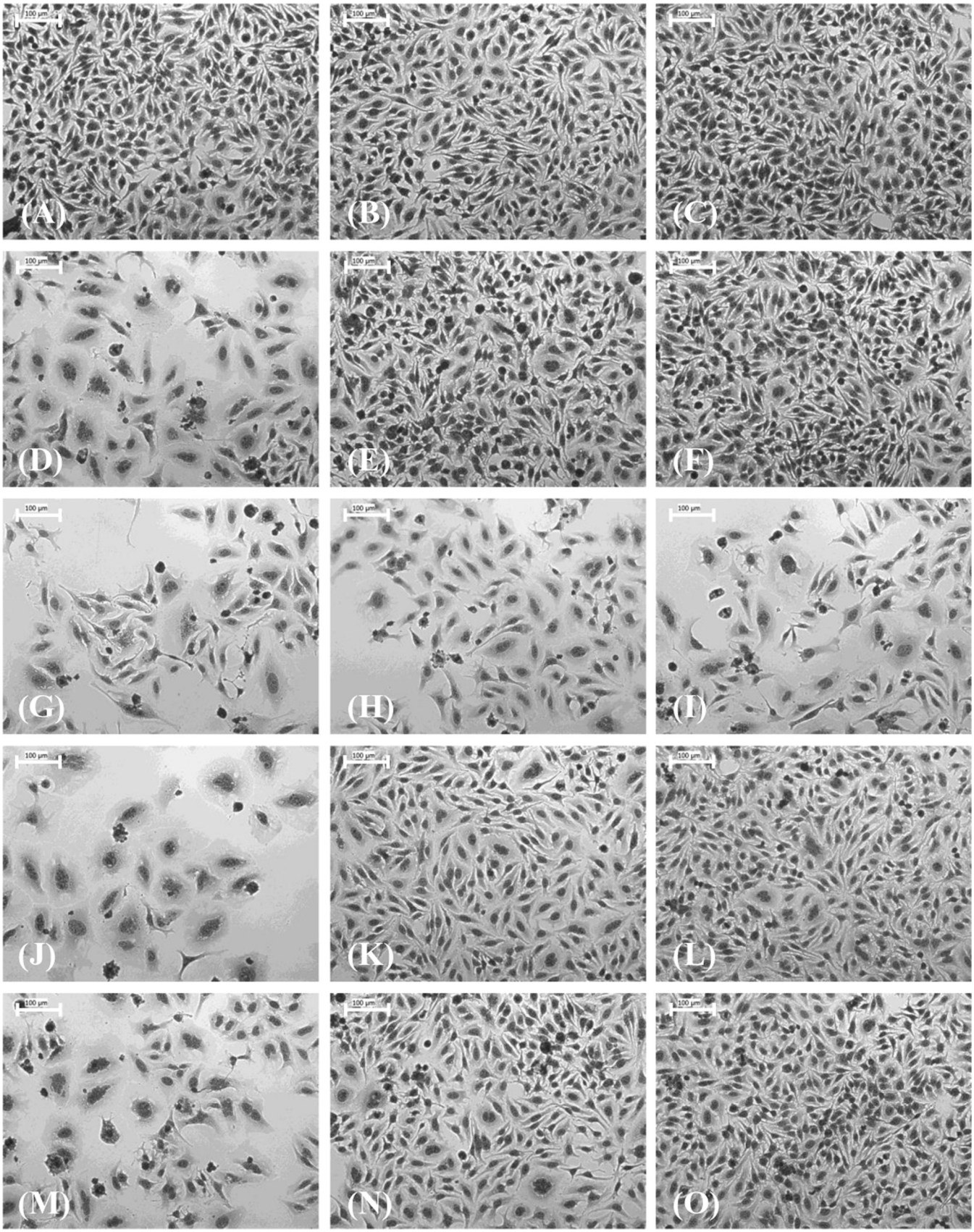

FIG 4 Neutralization of CDT activity by anti-rChCdt-II subunit antibody. HeLa cells were treated with PBS (A to C) or with sonic lysate of bacterial strains: (D to F) *E. coli* cells carrying the TH-pET28(a) vector with the *cdt-IIABC* genes; (G to I) *C. jejuni* strain 81-176; (J to L) *C. hyointestinalis* strain ATCC 35217^T; (M to O) *C. hyointestinalis* strain Ch022. HeLa cells were preincubated at 37°C for 1 h with PBS (A, D, G, J, and M) or with antibody against either rChCdt-IIA (B, E, H, K, and N) or rChCdt-IIC (C, F, I, L, and O), and incubation was continued for 72 h. Scale bars correspond to 100 μm. Magnification, ×100.

Distribution of the *cdt-II* gene cluster. The distribution of the *cdt-II* gene was examined by colony hybridization assay using 23 *C. hyointestinalis* strains isolated from animals and 1 human isolate. The *cdt-IIA*, *cdt-IIB*, and *cdt-IIC* gene probes prepared from *C. hyointestinalis* ATCC 35217^T hybridized with all *C. hyointestinalis* strains tested (Table 1). None of these probes reacted with *E. coli* strain C600, which was used as a negative control. The *cdt-IIB* gene probe did not hybridize with any other *Campylobacter* species tested in this study (Table 1), indicating that *cdt-II* genes are specifically present in *C. hyointestinalis*.

DISCUSSION

In recent years, increasing numbers of *C. hyointestinalis* organisms have been isolated from various animal sources, including pigs, and from diarrheal patients, as well as from immunocompromised hosts (1–13). However, to date no information is available about virulence factors and virulence mechanisms of *C. hyointestinalis* isolated from animals. Here, we show for the first time that *C. hyointestinalis* isolated from animals such as pigs and cows produces a new variant of biologically active CDT (ChCDT-II) which is immunologically different from ChCDT-I (Fig. 3) produced by a clinical isolate of *C. hyointestinalis* strain Ch022 (16). Interestingly, most of the animal-originated strains of *C. hyointestinalis* carry both gene clusters coding for ChCDT-I and ChCDT-II (Table 1). In addition, we have shown that the strain Ch022, isolated from a diarrheal patient in Thailand, also contained both *cdt-I* and *cdt-II* genes (Table 1). To test whether *C. hyointestinalis* strains carrying both gene clusters *cdt-I* and *cdt-II* have more cytotoxic activity than strains carrying only the *cdt-II* genes, the CDT titer of these strains was determined by using HeLa cells (Table 1). CDT titers of the strains positive for only *cdt-II* were the same or higher than those of the *C. hyointestinalis* strains carrying both gene clusters *cdt-I* and *cdt-II* (Table 1). At present, we cannot explain why strains positive for only *cdt-II* showed more cytotoxic effects than the strains containing both clusters, i.e., *cdt-I* and *cdt-II*. The cytotoxic activity of *C. hyointestinalis* strain ATCC 35217^T or Ch022 was not neutralized by the antibody against rChCdt-IIB protein alone or against both proteins rChCdt-IB and rChCdt-IIB, respectively (data not shown). However, the cytotoxic activity of *C. hyointestinalis* strains (ATCC 35217^T, Ch022, SS, 2033, and 2038) was clearly neutralized by the antibodies against rChCdt-IIA and rChCdt-IIC (Fig. 4M to O and data not shown) regardless of copy number of *cdt* genes. These data suggest that ChCDT-II could be dominantly produced from strains, such as Ch022 and 2033, possessing both the *cdt-I* and *cdt-II* gene clusters.

Interestingly, CdtBs produced by *C. jejuni* and *C. lari* were immunologically related despite production of this protein by different species (26). However, ChCdt-IB and ChCdt-IIB were immunologically unrelated, although these toxins are produced not only by the same species but also by the same strain (Fig. 3). At present, the basis of this immunological difference between these similar proteins is unknown and warrants further studies.

Recently, we have determined the entire nucleotide sequence of the *cdt* genes (*cdt-I*) of the *C. hyointestinalis* strain Ch022 isolated from a diarrheal patient in Thailand and demonstrated that the strain produced a biologically active CDT with unique amino acid sequences (16). A PCR-RFLP assay developed recently was not able to amplify the *cdt-IB* gene in 3 *C. hyointestinalis* strains isolated from animals (22). In this study, initially to confirm whether or not the *cdt-IB* gene-negative *C. hyointestinalis* strains produce CDT, the filter-sterilized lysates of the three strains were incubated with HeLa cells. Indeed, CDT activity was demonstrated by all apparently *cdt-IB* gene-negative *C. hyointestinalis* strains (Fig. 1A to C). To determine whether or not a *cdt-IB* gene variant is present in these strains, a colony hybridization assay was carried out with 24 *C. hyointestinalis* strains (23 of animal and 1 of human origin) using *cdt-IB* as a gene probe. In agreement with the result obtained by the PCR-RFLP assay, the *cdt-I* gene cluster was not detected in 3 *C. hyointestinalis* strains out of 24 tested (Table 1). We then selected *C. hyointestinalis* strain ATCC 35217^T, which was isolated from swine with proliferative enteritis (1, 2), as a representative *cdt-IB* gene-negative strain for further study and attempted to amplify the *cdtB* gene by degenerative primers as reported previously (15, 25). Accordingly, we successfully amplified the *cdtB* homologous gene and determined the entire sequences of *cdt* genes. Interestingly, nucleotide sequence homology was found to be low between *cdt-II* and *cdt* genes of *Campylobacter* species, including *cdt-I* (49.5 to 55.2%). The genes related to prophage were found in the upstream region of the *cdt-II* gene cluster (ORFs 6 to 10 in Fig. 2; also see Table S3 in the supplemental material), although none of these genes related to horizontal gene transfer was found in the flanking regions of the *cdt-I* gene cluster, indicating that the *cdt-II* genes in *C. hyointestinalis* strain ATCC 35217^T are acquired by horizontal gene transfer. Another interesting finding of this study is that *cdt-II* genes are present in all 24 *C. hyointestinalis* strains isolated from animals and human (Table 1). To the best of our knowledge, this is the first report showing that 2 different variants of *cdt* genes are present in a strain of the genus *Campylobacter*. Most *C. hyointestinalis* strains used in this study have been isolated from healthy gorilla, monkey, elephant, and cow. On the other hand, the *C. hyointestinalis* strains ATCC 35217^T and 1-1 were isolated from pigs with proliferative enteritis, and the strain SS was isolated from pork (Table 1), suggesting that *C. hyointestinalis* is an important zoonotic pathogen between pigs and humans.

A PCR-RFLP assay has been developed for the detection and differentiation of seven *Campylobacter* species, including *C. hyointestinalis* (22). Because common primers were designed from the conserved regions of the *cdt* genes of *C. jejuni*, *C. coli*, and *C. fetus* (18), the detection limit and sensitivity of *C. hyointestinalis* were lower than those of other campylobacters. Three or six mismatches were found in the reverse primer compared to the *cdt-I* gene or *cdt-II* gene, respectively (data not shown). Out of 24 *C. hyointestinalis* strains, PCR product was not obtained from 3 strains because these strains were *cdt-I* gene negative (Table 1). Since the *cdt-II* genes are present in all *C. hyointestinalis* strains analyzed in this study, the *cdt-II* genes may be appropriate marker genes to identify *C. hyointestinalis*. It is expected that the redesigning of primers on the basis of the nucleotide sequence of the *cdt-IIB* gene reported in the present study will increase the sensitivity of the PCR-RFLP assay to detect strains carrying this newly identified virulence locus.

Among 25 species of campylobacters, *C. jejuni* and *C. coli* are the most frequently isolated *Campylobacter* species and most studied species (13). One of the reasons for the high detection level of these *Campylobacter* species might be the availability of suitable culture methods for these organisms. Because antimicrobial agents such as cephem antibiotics often are used in selective media, some campylobacters are difficult to isolate by currently avail-

able methods. When PCR-based detection methods as well as nonselective media and hydrogen were employed, increasing numbers of *C. hyointestinalis* organisms were isolated from diarrheal patients, in particular from children and immunocompromised hosts (4, 7–12). For this reason, the development of rapid detection methods for clinically important campylobacters, including *C. hyointestinalis*, is required (22, 27). The evaluation of the clinical significance of *C. hyointestinalis* infection to humans and animals, particularly swine with proliferative enteritis, is needed. Similarly, further studies are needed to elucidate the pathogenic mechanism(s) of *C. hyointestinalis*. The identification of virulence factors is the first step to understanding its pathogenesis. Future studies are needed to determine the importance of CDT in the pathogenesis of *C. hyointestinalis*, and more emphasis should be given to identify a new virulence factor(s) of this pathogen and its contribution to pathogenesis, if any.

In conclusion, we have identified a new *cdt* variant gene, called *cdt-II*, in the genomes of *C. hyointestinalis* strains isolated from swine with proliferative enteritis. The *cdt-II* gene cluster, encoding the novel variant ChCDT-II protein, seems to be immunologically unique from ChCDT-I. ChCDT-II may be a virulence factor of *C. hyointestinalis* associated with animal diseases. The *cdt-II* genes have low homologies with *cdt-I* and *cdt* genes of other *Campylobacter* species. Most of the *C. hyointestinalis* strains isolated from animals carry two *cdt* variant gene clusters, *cdt-I* and *cdt-II*. The *cdt-II* genes may be ubiquitously present in *C. hyointestinalis* in a species-specific manner and may be a good target for species identification.

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