

Cytolethal Distending Toxin Type I and Type IV Genes Are Framed with Lambdoid Prophage Genes in Extraintestinal Pathogenic *Escherichia coli*[∇]

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Five types of cytolethal distending toxin (CDT-I to CDT-V) have been identified in *Escherichia coli*. In the present study we cloned and sequenced the *cdt-IV* operon and flanking region from a porcine extraintestinal pathogenic *E. coli* (ExPEC) strain belonging to serogroup O75. We confirmed that similar to other CDTs, CDT-IV induced phosphorylation of host histone H2AX, a sensitive marker of DNA double-strand breaks, and blocked the HeLa cell cycle at the G₂-M transition. The *cdt-IV* genes were framed by lambdoid prophage genes. We cloned and sequenced the *cdt-I* operon and flanking regions from a human ExPEC O18:K1:H7 strain and observed that *cdt-I* genes were also flanked by lambdoid prophage genes. PCR studies indicated that a gene coding for a putative protease was always associated with the *cdtC-IV* gene but was not associated with *cdtC* genes in strains producing CDT-I, CDT-III, and CDT-V. Our results suggest that the *cdt-I* and *cdt-IV* genes might have been acquired from a common ancestor by phage transduction and evolved in their bacterial hosts. The lysogenic bacteriophages have the potential to carry nonessential “cargo” genes or “morons” and therefore play a crucial role in the generation of genetic diversity within ExPEC.

Cytolethal distending toxins (CDTs) were the first bacterial toxins that block the eukaryotic cell cycle that were described, and they suppress cell proliferation and eventually lead to cell death. CDTs represent an emerging toxin family, which was initially described for *Escherichia coli* but is actually widely distributed among a variety of pathogenic bacteria, including *Shigella dysenteriae*, *Salmonella enterica* serovar Typhi, *Campylobacter* spp., *Helicobacter* spp., *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetecomitans*, and *Haemophilus ducreyi* (31, 34).

A CDT is a tripartite holotoxin (28) in which CdtB is the active subunit and CdtA and CdtC form a heterodimeric subunit apparatus required for delivery of CdtB into the cell (8, 23, 24). The toxin of *S. enterica* serovar Typhi is an exception. This organism does not encode CdtA or CdtC; instead, the enzymatic CdtB is delivered directly into host cells by a bacterial internalization pathway (14). Nuclear entry of CdtB, which relies on an atypical nuclear localization signal, is crucial for the cytotoxic activity (25, 27, 29). CdtB has DNase I-like activity (10, 15, 23). Once in the host cell nucleus, CdtB induces DNA double-strand breaks in both proliferating and nonproliferating cells (13, 15, 26). Although pathogenic roles of CDT

have been shown for chronic infection in mouse models (12, 39), CDT has not played a significant role in acute infection models tested to date (25, 40, 48, 49). Nevertheless, CDT appears to be a virulence factor; accordingly, studying its spread is important.

So far, five different CDTs have been reported for *E. coli*, and they were designated in order of publication. CDT-I (44) and CDT-II (38) were identified in enteropathogenic *E. coli* (EPEC) serotype O86:H34 and O128:NM strains, respectively. CDT-III was cloned from an *E. coli* serotype O15:H21 strain isolated from a septicemic calf and sequenced (36). CDT-III is encoded by pVir, a conjugative plasmid which also codes for another toxin, cytotoxic necrotizing factor type 2 (35). CDT-V was identified in the sorbitol-fermenting Shiga toxin (Stx)-producing *E. coli* (STEC) serotype O157:NM strain 493/89 (18) and was detected in several other non-O157 STEC strains (5). We recently identified and characterized CDT-IV in human- and animal-pathogenic *E. coli* strains of intestinal and extraintestinal origin (46); however, the complete sequence of the *cdt-IV* gene cluster was not determined. In the present study we confirmed the genotoxic activity of CDT-IV, sequenced the *cdt-IV* and *cdt-I* gene clusters and flanking regions, and investigated the dissemination of these clusters in different types of CDT-producing *E. coli* strains.

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MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study are listed in Table 1. The CDT-I-producing prototype strain E6468/62 (serotype O86:H34) was kindly provided by James B. Kaper. CDT-V-producing reference strain 493/89 and CDT-V producing strains 702/88 and 703/88 (serotype O157:NM) were kindly provided by Helge Karch.

TABLE 1. *E. coli* strains used in this study and occurrence of two *cdt-IV* flanking genes (*orf5* and *rorf1*) of ExPEC strain 28C and their association with *cdt* genes in CDT-producing *E. coli* strains

Strain	Origin	Serotype or serogroup	Genotype	<i>cdt</i> type	<i>orf5</i>	<i>orf6</i>	<i>orf5/cdtA</i>	<i>rorf1</i>	<i>cdtC/rorf1</i> ^a	Reference
E6468/62^d	Human	O86:H34	<i>eae cdt</i>	<i>I</i>	–	–	–	–	–	44
ICC95	Human	O86	<i>eae cdt</i>	<i>I</i>	–	–	–	–	–	2
E990	Human	O86:NM	<i>eae cdt</i>	<i>I</i>	–	–	–	–	–	6
EF142	Pigeon	O128:NM	<i>eae cdt</i>	<i>I</i>	–	–	–	–	–	This study
ED177	Human	O157:H7	<i>eae stx₁ stx₂ cdt</i>	<i>I</i>	–	–	–	–	–	This study
S5	Bovine	O78	<i>cdt cnf2</i>	<i>III</i>	–	–	–	–	–	36
EF56	Human	O55:H21	<i>cdt cnf2</i>	<i>III</i>	–	–	–	–	–	This study
EF147	Roe deer	O26	<i>eae cdt cnf2</i>	<i>III</i>	–	–	–	+	–	This study
ED1451	Dairy cow	O88	<i>cdt</i>	<i>III</i>	–	–	–	+	–	This study
28c	Porcine	O75	<i>cnf1 cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
H173	Human	O2	<i>cdt</i>	<i>IV</i>	+	+	+	+	+	46
H83	Human	O75	<i>cnf1 cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
H62	Human	O75	<i>cnf1 cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
H78	Human	O75	<i>cnf1 cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
AI-9	Porcine	O164	<i>cnf1 cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
H193	Human	O170	<i>cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
H104	Human	O rough	<i>cnf1 cdt hly</i>	<i>IV</i>	+	+	+	+	+	46
H29	Human	O6	<i>cnf1 cdt hly</i>	<i>IV</i>	–	–	–	+	+	46
E250	Poultry	O115	<i>cdt</i>	<i>IV</i>	–	–	–	+	+	46
E253	Poultry	O115	<i>cdt</i>	<i>IV</i>	–	–	–	+	+	46
AII-40	Porcine	O6	<i>cnf1 cdt hly</i>	<i>IV</i>	–	–	–	+	+	46
H155	Human	O rough	<i>cdt</i>	<i>IV</i>	–	–	–	+	+	46
493/89^b	Human	O157:NM	<i>eae stx₂ cdt</i>	<i>V</i>	–	–	–	+	–	5
703/88 ^b	Human	O157:NM	<i>eae stx₂ cdt</i>	<i>V</i>	–	–	–	+	–	This study
702/88 ^b	Human	O157:NM	<i>eae stx₂ cdt</i>	<i>V</i>	–	–	–	+	–	This study
C600 ^c	Laboratory	K-12			–	–	–	–	–	42

^a The *cdtC-III/V-s-rorf1-as1* (Table 2) and P105(5)/*rorf1-as2* primer pairs were used for detection of the potential association between *cdtC* and *rorf1* in strains EF147, ED1451, 493/89, 703/88, and 702/88.

^b *E. coli* O157:NM strains 493/89, 703/88, and 702/88 strains were kindly provided by H. Karch, University of Münster, Münster, Germany.

^c *E. coli* C600 was used as a negative control in all PCRs.

^d Bold type indicates *cdt* reference strains.

DNA techniques, PCR, and nucleotide sequencing. Cosmid libraries were constructed from CDT-IV-producing extraintestinal pathogenic *E. coli* (ExPEC) strain 28C (serogroup O75) (9, 46) and from CDT-I producing ExPEC strain IHE3034 (serotype O18:K1:H7) (21, 30). Both libraries were created by using the same strategy. Briefly, genomic DNA samples were partially digested with *Sau3AI* and cloned into the *Bam*HI sites of the SuperCos-1 vector (Stratagene). Following in vitro packaging and introduction into *E. coli* XL1-Blue, about 2,000 ampicillin-resistant colonies of each library were collected. The libraries were screened by PCR using *cdtB-IV-* and *cdtB-I-*specific primers, respectively (46). The CDT production of the PCR-positive *cdt-IV* and *cdt-I* clones was investigated by using nonconfluent HeLa cell monolayers in 96-well plates as described previously (35, 36).

Typing of *cdt* genes was conducted by using previously described PCR primers and assay conditions (5, 46). The dissemination of *cdt-IV* flanking genes in CDT-producing strains and their association with *cdt* genes were investigated by performing PCR with primers listed in Table 2. The positions of these PCR primers are shown in Fig. 3.

For sequencing, DNA samples from a CDT-IV clone and a CDT-I clone were purified with a QIAquick genome purification kit (Qiagen) and used as templates for sequencing with an ABI PRISM 377 automated sequencer. Sequencing was performed by the chain termination method with a dye terminator kit (Applied Biosystems) using T3, T7, or synthetic primers.

Searches for open reading frames (ORFs) and prediction of translation start positions were performed by using Vector NTI software.

Searches for homologous DNA sequences were conducted using BLAST software (3) and the GenBank database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Nucleotide sequences of *cdtABC* genes with all the available *E. coli cdt* alleles and the *cdt* genes of one *Campylobacter* reference strain were aligned by using the ClustalW multiple-sequence alignment program (45). The phylogenetic analysis was carried out with the MEGA (Molecular Evolutionary Genetics Analysis) 3.1 program (22) using the neighbor-joining method (41). Distances were calculated using the Kimura two-parameter nucleotide substitution model with pairwise deletion of gaps. The branches were tested by bootstrap analysis

(11) of 300 replicates. The tree was rooted with the *cdtABC* sequences of *Campylobacter jejuni* M1221 (accession number NC_003912).

CDT-IV genotoxic effects. Experiments and preparation of bacterial lysates were performed as described previously (30, 36). Briefly, *E. coli* strains were grown at 37°C in tryptic soy broth with vigorous (200 rpm) shaking for 2 days. Bacterial cells were sonicated, and the sonic lysates were sterile filtered separately by using 0.22- μ m-pore-size filters. HeLa cells were treated with sterile sonic lysates of CDT-IV-producing *E. coli* strain 28C. After 72 h of interaction at 37°C in a 5% CO₂ atmosphere, the infecting material was removed by washing the HeLa cell monolayers several times. The morphological changes characteristic of CDT were investigated after staining with anti-phospho-histone H2AX (γ H2AX) antibodies (Cell Signaling Technology), followed by rhodamine-conjugated secondary antibodies, or with Giemsa stain, as described previously (30). The cell cycle distribution of 20,000 cells was determined as described previously (36) by flow cytometry. Lysates of CDT-I-producing strain E6468/62 were used as positive controls, and lysates of *E. coli* C600 were used as negative controls.

Nucleotide sequence accession number. The sequence of the *E. coli* 28C *cdt-IV* gene cluster and neighboring region (see below) has been deposited in the GenBank database under accession number AY578329.

RESULTS AND DISCUSSION

Cloning and genotoxic activity of CDT-IV from ExPEC strain 28C. To clone *cdt-IV* genes, a cosmid library was constructed using porcine *E. coli* strain 28C (9), in which the CDT-IV toxin was identified for the first time (46). The library was screened by PCR using *cdtB-IV*-specific primers (46), and CDT-IV production of the PCR-positive clones was tested by using HeLa cell cultures. Sonic lysates of PCR-positive clones altered HeLa cells like sonic lysates of parental strain 28C and

TABLE 2. PCR primers used in this study

Primer	Target	Nucleotide sequence	Position	Size (bp)	GenBank accession no.	Reference
orf5-s1 orf5-as1	orf5	5'-GGGAGACATCACCACGCTAAAG-3' 5'-TCCTACTTCTCGGCAATCAATC-3'	4028–4049 4456–4478	450	AY578329	This study
orf5-s2 cdtA-IV-as1	orf5/orf6/ <i>cdtA-IV</i>	5'-GCTAACGAAGATGATGCACC-3' 5'-CCATTTGAAGCAACAGCGAC-3'	4512–4531 5625–5643	1,131	AY578329	This study
orf5-s2 cdtA-IV-as2	orf5/orf6/ <i>cdtA-IV</i>	5'-GCTAACGAAGATGATGCACC-3' 5'-ATCACCCCGTTTTTAAAGGC-3'	4512–4532 5874–5893	1,381	AY578329	This study
cdtC-IV-s1 rorf1-as2	<i>cdtC-IV</i> /rorf1	5'-GAGCTGTGCAAATCAAGTCG-3' 5'-GAGAAAATCCCGAAAAGACG –3'	7418–7437 8031–8050	632	AY578329	This study
rorf1-s1 rorf1-as1	rorf1	5'-TGACAAATCCCCTGTGAGAAGTGGC-3' 5'-CCGTGGCGTATTCGTATGGAACAC-3'	8163–8186 8564–8541	401	AY578329	This study
cdtC-III/V-s	<i>cdtC-III</i> / <i>cdtC-V</i> /rorf1	5'-ATCAAAAGTGTCTGTCTGG-3'	7168–7187 3061–3080 2089–2109 2089–2109 1997–2016	1,396 ^a	AY578329 U89305 AJ508930 AY365045 AY365042 AY365044	This study
rorf1-as1		5'-CCGTGGCGTATTCGTATGGAACAC-3'	8564–8541		AY365044	This study
c338f c2135r	<i>cdtA-V</i>	5'-AGCATTAATAAAAAGCACGA-3 5'-TACTTGCTGTGGTCTGCTAT-3'		1,329	AY578329	5
P105 c2767r	<i>cdtC-V</i>	5'-GTCAACGAACATTAGATTAT-3' 5'-ATGGTCATGCTTTGTTATAT-3'		748		5

^a Predicted size, calculated on the basis of the position of the homologous sequence (14/20) in 28C (positions 7168 to 7187).

CDT-I-producing prototypic strain E6468/62. Three days following treatment, HeLa cells showed morphological changes that included cell body distension, giant nuclei, and G₂ cell cycle arrest. Similar to CDT-I, CDT-IV induced phosphorylation of the histone H2AX, a sensitive marker for double-strand DNA breaks. In contrast, a lysate of a laboratory *E. coli* strain did not cause morphological changes, cell cycle arrest, and H2AX phosphorylation (Fig. 1 and data not shown). This observation confirmed that like other CDTs, CDT-IV inflicted DNA damage in eukaryotic cells.

Comparison of nucleotide sequences of *cdt-IV* gene clusters in ExPEC strains 28C and APEC O1. The *E. coli* 28C *cdt-IV* gene cluster and neighboring region (see below) were sequenced (GenBank accession number AY578329). A similar *cdt-IV* operon (GenBank accession number NC008563) is also present in the recently sequenced genome of avian pathogenic *E. coli* serotype O1:K1:H7 strain APEC O1 (19). The nucleotide sequences of the *cdtA-IV*, *cdtB-IV*, and *cdtC-IV* genes are 99, 99, and 98% identical in strains 28C and APEC O1. Nucleotide sequences of the *cdtABC* genes for all deposited *E. coli* alleles were then aligned by using the ClustalW multiple-sequence alignment program (45). In order to facilitate the sequence comparison, we used homologous sequences of *cdtABC* from *C. jejuni* as an outgroup. The levels of homology between *cdtB* genes were higher than the levels of homology between the *cdtA* and *cdtC* genes, consistent with the hypothesis that CdtB is the active enzymatic subunit of the holotoxin (data not shown). A neighbor-joining tree based on the multiple-sequence alignments was constructed, and bootstrap values were generated (Fig. 2). Two different phylogenetic lineages were observed for *cdtABC* alleles from *E. coli*. The *cdt-I*

and *cdt-IV* genes appeared to belong to the same phylogenetic lineage, whereas the *cdt-II*, *cdt-III*, and *cdt-V* genes clustered together in another lineage. These data suggest that the *cdt* genes were acquired by horizontal transfer events at some point and evolved separately since then.

***cdt-IV* gene clusters in ExPEC strains 28C and APEC O1 are framed by lambdoid prophage genes.** A DNA homology search indicated that the *cdt-IV* operon of strain 28C is flanked on both sides by prophage-related ORFs encoding putative prophage-related proteins. These putative proteins include a lambdoid prophage host specificity protein (orf1), a Lom-like protein (orf2), a putative tail fiber protein (orf3), a putative protease encoded in enterohemorrhagic *E. coli* (EHEC) EDL933 prophage CP-933 (rorf1), and a putative OmpT-like outer membrane protease (rorf2). Seven of the eight ORFs that were identified in the *cdt-IV* locus of *E. coli* strain 28C were also present in the APEC O1 strain, and all of them were grouped in the same way in these two strains (Fig. 3). As the APEC O1 genome was only partially annotated at the beginning of this study, the present nucleotide sequence analysis of *cdt-IV* flanking ORFs of strain 28C represents an alternative annotation for the *cdt-IV* locus in the APEC O1 strain. Sequence comparison of the *cdt* alleles in ExPEC strain 28C (CDT-IV), APEC O1 (CDT-IV), IHE3034 (CDT-I), and a recently described (4) inducible CDT-I-encoding lambdoid prophage (CDT-IΦ) genome in EPEC revealed that in all cases the *cdt* genes were located downstream of the prophage head and tail genomic regions. Between the structural-protein-encoding genes and the *cdt* genes there is a variable region that spans the tail fiber and orf6 genes (Fig. 3). The main differences between the sequences of strains 28C, APEC O1, and

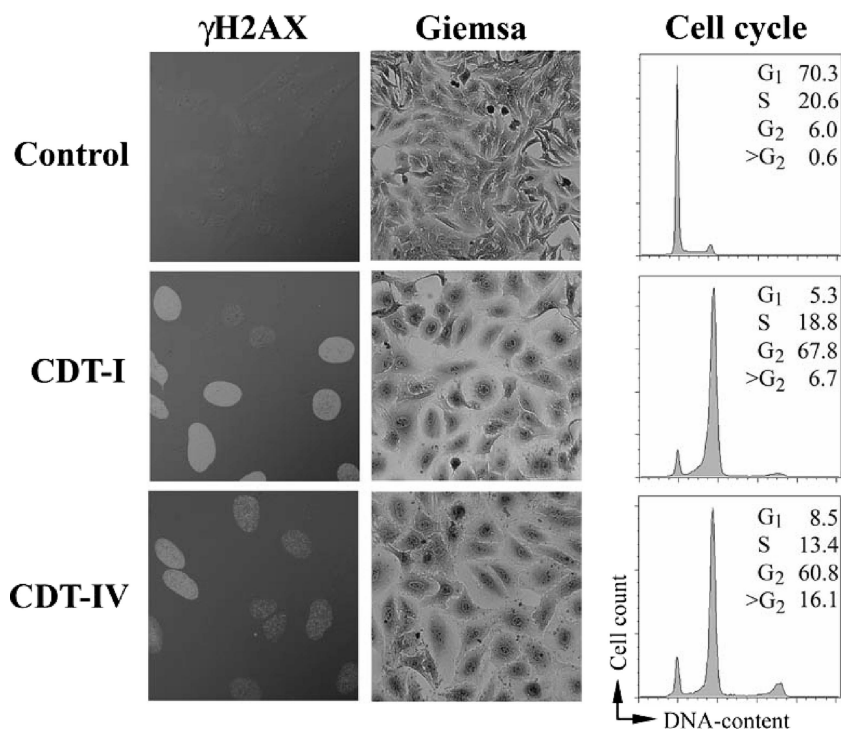


FIG. 1. CDT-IV genotoxic effects. HeLa cells were treated with sterile filtered sonic lysates of bacteria producing CDT-I (E6468/62) or CDT-IV (28C). Seventy two hours following treatment the cells were stained as described elsewhere (28) with anti-phospho-histone H2AX (γ H2AX) antibodies, followed by rhodamine red-conjugated secondary antibodies (left panels), or with Giemsa stain (middle panels). The cell cycle distribution of 20,000 cells was determined as described previously (34) by flow cytometry (right panels). The percentages of cells in each cell cycle phase are indicated. CDT-I- and CDT-IV-treated cells exhibited nuclear γ H2AX (indicating host DNA double-strand breaks), enlarged nuclei and cell bodies, and an absence of mitotic figures. The majority of CDT-I- and CDT-IV-treated cells had a 4n DNA content, indicating that these cells were blocked at the G₂-M transition in response to DNA damage.

IHE3034 were restricted to orf4 and orf5. orf4 is replaced by a 2-kb region in APEC O1 and by the c3149 ORF in IHE3034, while orf5 characterizes the CDT-IV 28C and APEC O1 ExPEC strains. The fact that the c3149 ORF and orf4 are similar but not identical could suggest that they have similar functions or the same function. The *cdt* downstream region is identical in CDT-IV strains, and the *cdtC* genes in the 28C and APEC O1 strains are flanked by two morons. These DNA sequence comparison data indicate that the CDT-IV-encoding locus may have undergone recombination events, resulting in exchange of DNA regions in different parts of the phage genome.

***cdt-I* gene cluster in ExPEC strain IHE3034 is flanked by lambdoid prophage genes.** On the basis of the known homology between *cdt-I* and *cdt-IV*, we decided to compare their flanking regions as well. However, the flanking sequences of the *cdt-I* operon in prototypic EPEC strain E6468/62 were not available (GenBank accession number U03293). Thus, we cloned and sequenced the *cdt-I* genes and their flanking regions in the CDT-I-producing O18:K1:H7 ExPEC strain IHE3034 (21, 30). A genomic DNA library was constructed and screened by PCR for the *cdtB-I* gene. From the PCR-positive *cdtB-I* clones, one clone producing CDT-I was selected for sequencing. An 18.5-kb DNA fragment was sequenced. Nucleotide sequence comparison revealed that the *cdtABC* genes of strain IHE3034 were 99% identical to the genes of EPEC prototypic strain E6468/62. The *cdt-I* genes in strain IHE3034 were also flanked by prophage-related ORFs. The *cdt-I*-flanking ORFs were homologous to several flanking

ORFs identified in the *cdt-IV* loci of strains 28C and APEC O1. Upstream of the *cdtA-I* gene three 28C-related genes, orf2, orf3, and orf6, encoding the putative Lom-like and tail fiber proteins and a CFT073-like hypothetical protein (c3147), were localized (Fig. 3). Downstream of the *cdtC-I* gene a P4-like prophage integrase gene (*intB*) was identified. This integrase gene was previously found in EHEC serotype O157:H7 reference strains EDL933 and Sakai and was found in the APEC O1 genome as well (16, 19, 37). However, *intB* was not associated with the *cdt-IV* operon in the APEC O1 strain.

A database search conducted with all other *cdt-I* flanking ORFs of strain IHE3034 revealed that the sequenced fragment contained only prophage genes. Furthermore, the nucleotide sequence comparison allowed further annotation of the *cdt-IV* locus in APEC O1. The prophage genes upstream of the *cdt-I* and *cdt-IV* operons encode several putative tail, minor tail, and tail assembly proteins. These genes are homologous and grouped similarly in IHE3034 and APEC O1. Interestingly, the last six genes at the left end of the IHE3034 fragment correspond to six genes which so far have been observed only in APEC O1 (data not shown).

Comparison of the *cdt-I* and *cdt-IV* genes and their flanking regions revealed that not only are these *cdt* operons similar but the *cdt* upstream flanking regions contain similar prophage genes. These *cdt* genes and their flanking genes might have been acquired from a common ancestor and after chromosomal integration in different bacteria could have evolved in slightly different ways, resulting in minor sequence variations and some deletions.

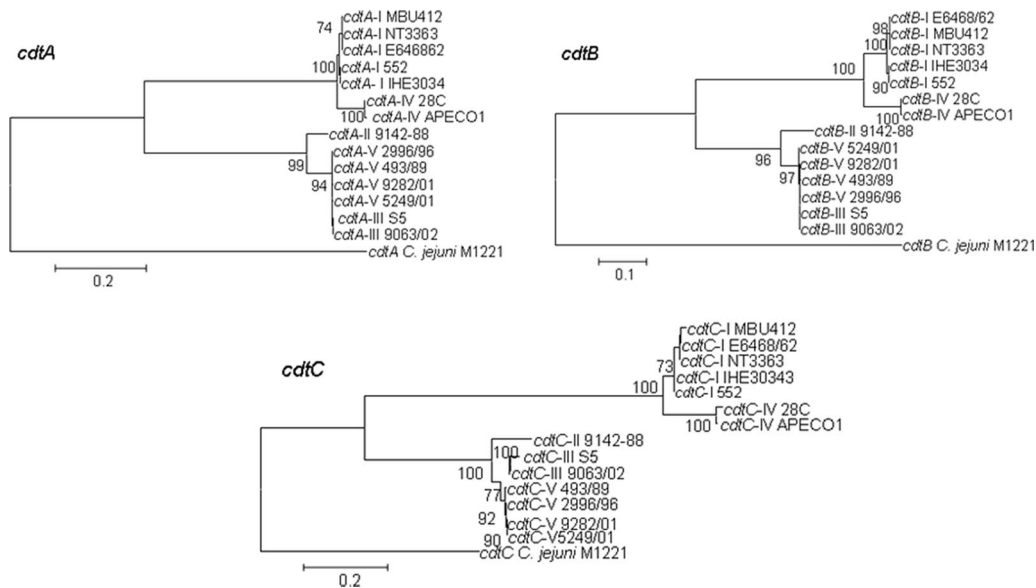
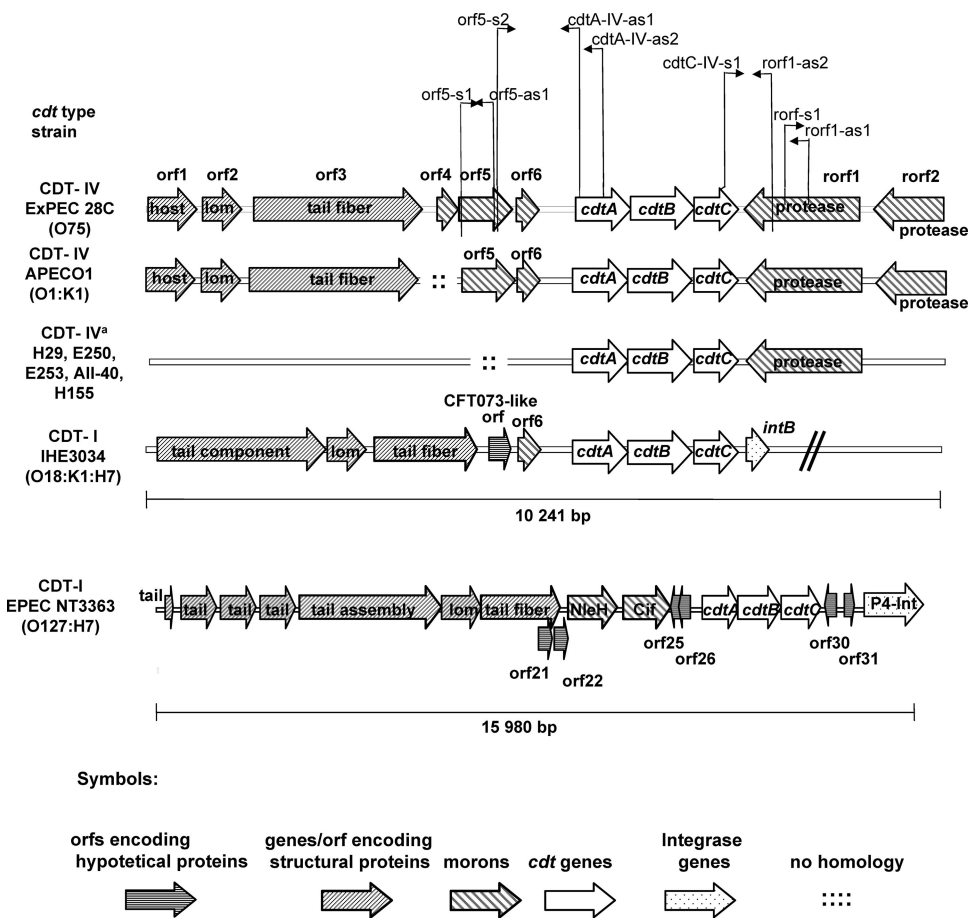


FIG. 2. Phylogenetic trees for *E. coli* *cdtABC I-V* and *C. jejuni* *cdt* genes. The neighbor-joining trees were constructed with MEGA3.1 using the Kimura two-parameter nucleotide substitution model with pairwise deletion of gaps. The levels of bootstrap support for the branches were calculated using 300 replicates, and only values greater than 70% are indicated at the nodes. The alignments of the *cdtA*, *cdtAB*, and *cdtAC* genes were 707, 812, and 582 nucleotides long, respectively. The bars indicate 10 and 20 differences in 100 nucleotides. The following reference sequences were used: for CDT-I, sequences of *E. coli* E6468/62 (serotype O86:H34; accession number U03293) and 552 (serotype O2:H12; accession number AB258385), CDT-I phage in EPEC strain NT3363 (accession number AB285204), and MBU 412 (Ont; accession number AF373206); for CDT-II, *E. coli* 9142-88 (serotype O128:NM, accession number U04208); for CDT-III, *E. coli* S5 (serotype O115:K?:H21; accession number U89305) and *E. coli* 9063/02 (O153:H18, accession number AY365044); for CDT-IV, *E. coli* 28C (serotype O75:K95; accession number AY578329) and *E. coli* APEC O1 (serotype O1:K1; accession number CP000468); and for CDT-V, *E. coli* 2996/96 (serotype O73:H18; accession number AY365045), *E. coli* 9282/01 (serotype O91:H21; accession number AY365042), *E. coli* 5249/01 (serotype O113:H21; accession number AY365043), *E. coli* 493/89 (serotype O157:NM; accession number AJ508930), and *C. jejuni* M1221 (accession number NC_003912).

Similarly to our findings, Janka et al. (18) previously demonstrated that in EHEC O157:NM strain 493/89 the *cdt-V* operon is framed by P2 prophage sequences. However, the *cdt-IV* and *cdt-I* flanking genes are not related to P2 phage genes.

Recently, an inducible lambdoid phage (CDT-1 Φ) which codes for CDT-I in EPEC strains was sequenced (4). The genome of this CDT-I-converting phage comprises 47,021 nucleotides in 60 predicted ORFs, which are organized into six genomic regions coding for the head and tail, passenger virulence traits (morons), and integrase, as well as unknown regulatory and lysis functions. The CDT-1 Φ genome has a highly mosaic structure and shows homology with sequences derived from several lambdoid prophages, including the UTI89 prophage of uropathogenic strains and an EPEC B171 prophage-like element of an EPEC and the serotype-converting SfV phage of *Shigella flexneri* (4). However, none of these phages have a *cdt* gene cluster in their passenger regions. Nonetheless, CDT-1 Φ and the other prophages are very similar in their overall genomic organization. The nucleotide sequence data for *E. coli* 28C and IHE3034 did not allow identification of the integration sites of the CDT-I and CDT-IV phages, but the genome sequence of the APEC O1 strain (19) revealed that the *cdt-IV* operon is framed by two prophages and pathogenicity island-associated DNA sequences, including integrase and tRNA genes. In the present study we also observed genetic mosaic organization among the sequenced *cdt-I* and *cdt-IV* alleles carried by ExPEC strains IHE3034 and 28C, respectively. This phenomenon could also be a result of extensive genetic exchanges among different phages, which might even occur in the mammalian intestine.

Dissemination of two *cdt-IV* flanking genes in CDT-producing strains. We decided to investigate the presence of two 28C-specific flanking prophage genes and their association with *cdt* genes in a set of CDT-producing *E. coli* strains. We used a PCR-based strategy (Fig. 3) with primers listed in Table 2. Five of the 25 CDT-producing *E. coli* strains investigated carried *cdt-I* genes, 4 strains carried *cdt-III* genes, 3 strains had *cdt-V* genes, and 13 strains harbored *cdt-IV* genes (Table 1). *orf5* codes for a hypothetical protein, which was detected only in eight *cdt-IV* strains, while *rorf1* encoding a putative protease similar to ECs1662 in EHEC strain Sakai was detected in all the *cdt-IV* strains. The *rorf1* sequences were also present in two additional *cdt-III* strains and three additional *cdt-V* strains. *orf5* was uniformly associated with *cdtA*, and the size of the *orf5/cdtA*-specific PCR amplicon indicated that all the *cdt-IV* strains carried *orf6* as well. In all the *cdt-IV* strains *rorf1* was located directly downstream of the *cdtC* gene. At the same time *cdtC* genes were not immediately upstream of *rorf1* in either the *cdt-III* strains or the *cdt-V* strains. These results indicate that the protease gene could characterize not only the *cdt-IV* strains, although this gene is always and exclusively adjacent to the *cdtC* gene in *cdt-IV* strains. The fact that *orf5* can be absent in some *cdt-IV* strains demonstrates that the *cdt-IV* prophages could be divergent. PCR results indicated that there was a progressive loss of upstream flanking genes, while no losses for the downstream prophage genes were observed in the *cdt-IV* region. Nucleotide sequence analysis verified these observations and revealed further sequence variations in CDT-encoding phages. The DNA fragment of *E. coli* strain IHE3034 showed great homology to CDT-IV-encoding



^a detected by PCR, these results are given in Table 1.

FIG. 3. Genomic comparison of *cdt* loci and flanking regions in *E. coli* 28C (*cdt-IV*), APEC O1 (*cdt-IV*), and IHE3034 (*cdt-I*) and CDT-IΦ. Searches for homologous sequences were performed by using BLAST software (3), and the results were used to align sequence data. The *cdt-I* and *cdt-IV* loci of strains IHE3034 and 28C were sequenced in the present study. The *E. coli* APEC O1 (accession number NC_008563) and lambdoid phage CDT-1Φ (accession number AB285204) sequences used were deposited previously in the database. Progressive loss of upstream flanking genes was observed in the *cdt-IV* region in strains H29, E250, E253, AII-40, and H155, as demonstrated by PCR, but no losses of the downstream flanking gene were observed. PCR primers for detection of *cdt* flanking genes present in *E. coli* 28C *cdt-IV* loci and their association with *cdt-IV* genes were designed. The PCR primer positions in the *cdt-IV* loci are indicated by arrows. The sequences of these primers and all other primers used are listed in Table 2.

phages, but in the corresponding fragment orf4 and orf5 were both deleted. In the APEC O1 strain an unrelated sequence was observed instead of orf4 between orf5 and orf3 (Fig. 3).

The orf4, orf5, orf6, *cdt*, CP-933-like protease (rorf1), and outer membrane protease (rorf2) genes can be defined as morons that are not required for the phage life cycle but may code for fitness factors contributing to the survival of the bacterial host (7). The fact that these “28C-specific” morons are also present in several intestinal *E. coli* and ExPEC strains, including uropathogenic isolates CFT073, 536, and UTI89, strain APEC O1, and EHEC strains EDL933 and Sakai, indicates that these morons, similar to virulence genes, might have spread by horizontal gene transfer (Table 3).

Phages in intestinal *E. coli* and ExPEC. CDT-I and CDT-II have been detected in *E. coli* strains that mainly cause human gastrointestinal infections. CDT-III and CDT-IV were identified in human- and animal-pathogenic *E. coli* strains of intestinal and extraintestinal origin, and these strains frequently

produced cytotoxic necrotizing factors (cytotoxic necrotizing factor type 1 and cytotoxic necrotizing factor type 2) and hemolysin as well. CDT-V was detected in *E. coli* O157 isolates and non-O157 STEC strains from humans with diarrhea or hemolytic-uremic syndrome (18). Although CDTs are produced by other diverse pathogenic bacterial species, the mechanism associated with possible horizontal transfer of the various *cdt* genes resulting in the wide distribution of *cdt* genes among pathogenic bacteria is not adequately known.

Bacteriophages are the major vehicles for transfer of genes, including fitness and virulence genes, between bacteria (7). Analysis of the genome sequences of EHEC O157:H7 strains Sakai and EDL933 revealed the presence of as many as 18 prophages (16, 37). The prophage DNA accounts for one-half of the 1.3 Mb of DNA found in O157 but is not present in the *E. coli* K-12 reference strain (32). Of the 18 prophages on the O157 Sakai chromosome, 13 are lambda-like phages, and in addition to Shiga toxins (Stx1 and Stx2), they encode other

TABLE 3. Morons of lambdoid prophage encoding CDT-IV in ExPEC strain 28C

ORF or gene	Feature or product of sequence	Start position	Stop position	Length (bp)	No. of identical nucleotides/ no. of nucleotides (% identity)	Position in clone or genome	Strain or location	GenBank accession no.	Reference
orf4	Hypothetical protein	3723	3992	270	270/270	3792–3992	ExPEC 28C	AY578329 ^d	This study
orf5	Conserved hypothetical protein	4005	4688	684	619/648 (95) 608/643 (94)	1512261–1512905 5046238–5046879	<i>E. coli</i> APEC O1	CP000468	19
orf6	Hypothetical protein	4699	4989	291	287/291 (98) 273/287 (95) 271/288 (94) 271/288 (94) 68/78 (87) 21/22 (95)	1512916–1513206 5046890–5047176 1220158–1220444 2052137–2051851 314824–314901 314784–314803	<i>E. coli</i> APEC O1	CP000468	19
					274/287 (95) 259/269 (96) 270/287 (94) 68/78 (87)	3022497–3022211 1371361–1371629 1446322–1446608 377759–377836	<i>E. coli</i> CFT073	AE014075	
					271/287 (94) 270/287 (94) 68/78 (87)	5029960–5030246 1282862–1283148 313235–313312	<i>E. coli</i> ÚTI 89	CP000243	
					270/287 (94) 68/78 (87) 21/22 (95)	1228776–1229062 369932–370009 369890–369911	<i>E. coli</i> 536	CP000247	
	Partial <i>sit</i> iron uptake system operon				269/287 (93)	4537–4251	<i>E. coli</i> chromosome	AM072350	
	Pathogenicity island II right junction				68/78 (87)	1131–1208	<i>E. coli</i> str4787	AY560916	
	Unknown genes <i>S. flexneri</i> 2a insertion sequence				62/61 (85) 21/22 (95)	20684–20744 20644–20663	<i>E. coli</i> str222	AY151282	
<i>cdtA</i>	CDT-IV A subunit	5502	6215	714	712/714 (99) 669/714 (93)	1513719–1514378 201–914	<i>E. coli</i> APEC O1 <i>E. coli</i> E6468/62	CP000468 U03293	19 44
<i>cdtB</i>	CDT-IV B subunit	6212	7033	822	821/822 (99) 722/822 (87)	1514429–1515250 911–1732	<i>E. coli</i> APEC O1 <i>E. coli</i> E6468/62	CP000468 U03293	19 44
<i>cdtC</i>	CDT-IV C subunit	7030	7602	573	563/573 (98) 437/496 (88)	1515247–1515819 1801–2296	<i>E. coli</i> APEC O1 <i>E. coli</i> E6468/62	CP000468 U03293	19 44
rorf1	Putative protease Encoded in prophage CP-933X	7672	9156	1485	1472/1485 (99) 1417/1485 (95) 1417/1485 (95)	1515889–1517373 1751300–1752784 1659506–1660990	<i>E. coli</i> APEC O1 <i>E. coli</i> O157:H7 EDL933 <i>E. coli</i> O157:H7 Sakai	CP000468 AE005174 BA000007	19 37 16
rorf2	Outer membrane 3b, protease VII	9341	10024	684	679/684 (99) 624/693 (90) 625/692 (90) 624/693 (90) 623/693 (89)	1517558–1518241 572657–573343 622998–623684 571059–571745 1661177–1661863	<i>E. coli</i> APEC O1 <i>E. coli</i> 536 <i>E. coli</i> UTI89 <i>E. coli</i> O157:H7 Sakai	CP000468 CP000247 CP000243 BA000007	19 16
	Omp precursor				623/693 (89) 620/692 (89)	1752971–1753657 629100–629786	<i>E. coli</i> O157:H7 EDL933 <i>E. coli</i> CFT073	AE005174 AE014075	37
	DLP12 prophage, outer membrane protease VII				620/693 (89)	583903–584589	<i>E. coli</i> MG1655	U00096	

virulence-related proteins, such as Lom homologues, Bor proteins, and zinc/copper-type superoxide dismutases (16). The *stx* genes are located in the genomes of heterogeneous, lytic (*stx*₂) or cryptic (*stx*₁) lambdoid phages (16, 37). The dissemination of Stx-encoding phages is the most likely mechanism for the emergence of new STEC serotypes that has been demonstrated in vitro (17, 43) and in vivo (1, 47).

Recently, Asakura et al. (4) provided evidence of the existence of an inducible converting *cdt-I* lambdoid phage (CDT-1Φ) in a serotype O127:H7 EPEC strain. It was demonstrated that the *cdt-I* gene cluster is transferred by CDT-1Φ to a recipient strain, which then produces biologically active CDT-I toxin. Interestingly, the virulence region of the CDT-1Φ genome contains, in addition to *cdt* genes, a truncated cycle-inhibiting factor gene (*cif*) and a type 3 secreted effector protein gene. Although several other EPEC strains belonging to serogroups O86, O142, and O127 were positive for the presence of *cdt* genes and produced biologically active CDT, only four of seven CDT-I strains investigated produced infectious CDT-1Φ particles. Southern hybridization analysis revealed the genetic diversity of CDT-I prophages carried by the different CDT-I-producing strains (4).

The regions encoding head and tail proteins and the lysis region in CDT-1Φ have homology to the regions in lambdoid Stx phages, but the locations of virulence regions in CDT-1Φ and Stx phages are significantly different. The *stx* genes are associated with the late regulatory region in the Stx phages, while the *cdt-I* and *cdt-IV* genes are not associated with the late regulatory genes.

Similar to intestinal pathogens, phages could play a fundamental role in the evolution of ExPEC strains. The O acetylation of the capsular polysaccharide is a phenomenon that has been known for a long time (33). Recently, an *E. coli* K1-specific virulent bacteriophage (CUS-3) carrying an O acetyltransferase gene, *neuO*, was described. Investigation of CUS-3 revealed that it is a 47,021-bp lambdoid phage, and the *neuO* gene is uniformly present in typically "high-virulence" *E. coli* O18 and O45 strains, while it is absent or rarely present in low-virulence strains or in strains belonging to less commonly isolated serotypes (20). If functional K1 and CDT-I phages are produced from the lysogenic ExPEC strains, other sensitive strains may be lysogenized by transduction. Lysogenization could lead to novel pathogenic strains harboring *neuO* and/or *cdt* genes (4, 20).

The locations of toxin genes in our sequenced *cdt-I* and *cdt-IV* alleles are substantially related to the location of the *cdt* gene cluster in CDT-1Φ. Although we do not have sequence information for a whole CDT-1Φ-like genome in ExPEC strains IHE3034 and 28C, our sequence data suggest that the ExPEC *cdt-I* and *cdt-IV* genes could be also parts of prophages. The overall G+C content of the *cdt-I* allele in IHE3034 is 51.51%, which is similar to that in *E. coli* (50%). The G+C content of the *cdt-I* genes is only 41.77%. The overall G+C content of the *cdt-IV* allele in 28C is 44.6%, and the G+C content of the *cdt-IV* genes is only 42.3%. These values indicate that both the *cdt-I* and *cdt-IV* genes might have been acquired recently by a perhaps a previously existing *E. coli* phage through multiple gene transfer events and that these genes evolved differently in their new bacterial hosts. Additionally, both of our sequenced *cdt* alleles contain several homologous structural genes upstream of the *cdt* genes, like those in the CDT-1Φ genome. Sequence comparisons indicated that in the *cdt-I* allele of IHE3034, similar to CDT-I, phage is also located

downstream of the head and tail region. One of the differences is that the virulence region in IHE3034 contains only the *cdt* genes, while in the CDT-1Φ virulence region there are other virulence genes, including a truncated *cif* gene and a non-locus-of-enterocyte-effacement-encoded type 3 effector gene, as well as (downstream of the *cdtC* gene) two other ORFs which encode putative hypothetical proteins (Fig. 3.). It is interesting that in the *cdt-I* allele of IHE3034 there is, upstream of *cdtA*, a 2.5-kb region which is missing from the genome of CDT-1Φ but is more or less present at the related position in the *cdt-IV* allele in 28C. In our sequenced *cdt-I* allele, the *cdtC* gene is associated with a P4-like integrase gene, while CDT-1Φ carries a different integrase gene downstream of the virulence region. The *cdtC-IV* genes in ExPEC strains 28C and APEC O1 are framed downstream by two prophage protease genes. The detailed mosaic structure observed for our *cdt-I* and *cdt-IV* alleles in ExPEC strains could also be the result of extensive genetic exchanges among different phages, as suggested by Asakura et al. for CDT-I phages carried by EPEC (4). These and similar recombination events might even occur in the human or animal intestine.

In summary, our results indicate that the *cdt-I* and *cdt-IV* genes might have been acquired by phage transduction from a common ancestor and that evolution of the CDT-encoding phages in different bacterial hosts generated differences in the *cdt* genes and their flanking DNA contents. More exact definitions of the differences in flanking regions would require further investigation. Similarly, the fact that the *cdt-II*, *cdt-III*, and *cdt-V* genes show significant homology despite their genetic location raises the possibility that the *cdt-II*, *cdt-III*, and *cdt-V* gene clusters might also have evolved from another ancestor and might have spread with mobile elements (perhaps plasmids) and evolved differently in the different bacterial hosts. The fact that *cdt-III* genes are located on a large conjugative virulence plasmid (pVir) in prototype strain S5 (36) could suggest the mode of acquisition from a common ancestor, but without sequence data it is impossible to predict the original donor of the *cdt-II*, *cdt-III*, and *cdt-V* genes. Further studies are required to answer all these questions.

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