Isolation and Characterization of an *Escherichia albertii* Strain Producing Three Different Toxins from a Child with Diarrhea

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SUMMARY: Here, we report a bacterium—isolated as the sole pathogen from a child with diarrhea—harboring *eae* and 2 different cytolethal distending toxin genes (*cdt*) that are homologous to *Escherichia coli* *cdt-I* and *cdt-II*. The bacterium was originally identified as atypical *E. coli* by conventional biochemical testing, but was finally identified as *E. albertii* by multilocus sequence analysis, which is the only method that can currently differentiate *E. albertii* from *E. coli*. The Shiga toxin 2f (*stx2f*) genes were also detected in the strain. Production of these 3 toxins was confirmed by western blotting and/or a cytotoxicity assay using eukaryotic cell lines. This is the first report showing the biological activity of CDT-I, CDT-II, and Stx2f in *E. albertii*.

INTRODUCTION

Diarrheagenic *Escherichia coli* can be classified into 6 categories, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli*, enteroinvasive *E. coli* enterohemorrhagic *E. coli*, enteroaggregative *E. coli* (EAEC), and diffuse adherent *E. coli* (*E. coli* DAD, *E. coli* DAD). However, other *E. coli* types, such as cytolethal distending toxin (CDT)-producing *E. coli* (CTEC) and EAEC heat-stable enterotoxin 1 (*astA*)-positive *E. coli*, have also been shown to be associated with diarrhea (2,3).

After the discovery of CDT in *E. coli* isolates from patients with diarrhea (4), CDT production has been reported in various other Gram-negative bacteria, including *E. albertii* (3,5,6). CDT is a tripartite bacterial toxin composed of 3 subunits, CdtA, CdtB, and CdtC. While CdtB, which has DNAse I activity, functions as the active subunit, CdtA and CdtC are thought to be responsible for binding of the holotoxin to unidentified receptor molecules on susceptible cells and entry of CdtB into the cytoplasm. CDT intoxication results in distortion and eventual death of certain cultured eukaryotic cell lines through the formation of DNA double-strand breaks leading to irreversible cell cycle arrest at G1 or G2. Although the importance of CDT production in vivo is unclear, it is thought to be associated with increased persistence, invasion, and disease severity (7–11).

In *E. coli*, 5 subtypes of CDT (I–V) have been described based on differences in their amino acid sequences (3). CTEC strains can produce any of the 5 CDT subtypes. However, during our surveillance of CTEC strains in stool specimens from pediatric patients with diarrhea (12) and healthy animals (13) by PCR-RFLP, ambiguous RFLP patterns were obtained. One such strain with an ambiguous pattern is an *E. coli* strain, isolated from healthy cattle, that contains the *cdt-III* and *cdt-V* genes (13), and the other is a *Providence alcalifaciens* strain harboring *cdt* genes (Pacdt) (5).

In this study, we isolated a bacterium containing *cdt-IB* and *cdt-IIIB* from a stool specimen of a pediatric patient with diarrhea during a CTEC surveillance program targeting children with gastrointestinal manifestations in Japan. The bacterium was initially identified as an atypical *E. coli*. However, extensive analysis finally showed that the bacterium was *E. albertii*. The *E. albertii* strain produced not only biologically active CDT-I and CDT-II but also Stx2f.

MATERIALS AND METHODS

Detection, isolation, and characterization of a *cdt*-gene-positive bacterium: A stool sample and rectal swab (specimen ID: P2660) were collected from a 1-year and 3-month-old child with diarrhea on August 2008 in Okayama, Japan. The stool specimen was tested for norovirus, adenovirus, and rotavirus with commercially available immune-chromatography kits at the hospital, and then tested for various bacteria, including *Campylobacter* spp., *Salmonella* spp., Shiga toxin-producing *E. coli* (STEIC), CTEC, and *Shigella* spp., at our laboratory by PCR targeting species- and pathotype-specific genes (12,14). These tests were conducted with and without enrichment culturing in tryptic soy broth (TSB).
with vigorous shaking or Preston broth under microaerobic conditions at 37°C for 18–24 h. The cdt genes were detected and subtyped by PCR-RFLP as described previously (12). A cdt gene-positive bacterium was isolated by a colony hybridization test using cdt-I and cdt-II gene-probes prepared as described previously (12). The cdt gene-positive bacterium was further characterized by conventional biochemical tests, an agglutination test (15) using 173 O antisera (O1-O173) prepared at the Osaka Prefectural Institute of Public Health (Osaka, Japan), and multilocus sequence analysis (MLSA) as described previously (13).

**Virulence gene profiling:** The presence of virulence genes specific to diarrheagenic E. coli (eae, bfpA, EAF, stx1, stx2, subAB, elt, est, aatA, astA, and invE) and necrotoxigenic E. coli (cnf1 and cnf2) was ascribed by PCR as described previously (13). The stx2 gene-subtype was examined by PCR as described previously (16).

**Sequencing of toxin genes:** The entire nucleotide sequences of cdt-I and cdt-II were analyzed as described previously (12). The stx2f gene-sequence was determined by sequencing a PCR product consisting of the partial stx2f genes and its flanking regions. Then, the flanking regions were sequenced by genome walking as described previously (17). The following primers were used to amplify the stx2f genes and determine the sequence of the upstream and downstream regions: Stx2f-5'-Ran1: 5'-CCTGAGATATATTTCCAAGNNNNN-3', Stx2f-Tar1: 5'-CCTGAGATATATTTCCAAG-3' and Stx2f-5'-Seq1: 5'-GCATAAGATGCTGGAAGAC-3', and Stx2f-3'-Ran1: 5'-AGAATACTGGCAAGACAGNNNNNN-3', Stx2f-3'-Tar1: 5'-AGAATACTGGCAAGACAG-3' and Stx2f-3'-Seq1: 5'-CTAATACCTGCAGTTACG-3'. The obtained sequences were analyzed with the DNA Lasergene software package (DNASTAR, Madison, WI, USA).

**Western blotting:** The P2660 strain was cultured in TSB at 37°C for 12 h. Bacterial cells were harvested from 1 mL of culture by centrifugation at 7,000 × g for 5 min, and then suspended in Dulbecco’s PBS to an optical density at 600 nm of 10. The cell suspension was boiled in non-reducing SDS sample loading buffer for 5 min, and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel. The separated proteins were blotted to PVDF membranes (Bio-Rad, Hercules, CA, USA) as described previously (5). The blots were incubated with rabbit anti-Cdt-IB and anti-PaCdtB sera, which were confirmed not to cross-react with each other, or with rabbit anti-Stx2fA serum and then with an HRP-linked goat anti-rabbit IgG antibody. The HRP signal was detected via chemiluminescence using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA). The anti-PaCdtB serum (5) could also bind to Cdt-IIIB due to its high shared amino acid sequence identity with Cdt-B (93.7%).

**Cytotoxicity assay:** The cytotoxicity assay was performed as described previously (13). Vero and CHO cells were cultured in Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 5% fetal bovine serum, GlutaMAX, and 1% antibiotic-antimycotic (100 ×; Thermo Fisher Scientific).

To neutralize the activities of CDT-I, CDT-II, and Stx2f, dilutions of a filer-sterilized lysate of strain P2660 were incubated with an equal volume of anti-Cdt-IB, anti-PaCdtB, and/or anti-Stx2fA sera for 30 min. These mixtures were then used in the cytotoxicity assay. Normal rabbit serum was used as a negative control.

**Nucleotide sequence accession numbers:** All nucleotide sequences obtained in this study have been deposited in the DDBJ database under accession numbers LC140875 (for cdt-I), LC140876 (for cdt-II), LC140877 (for stx2f), and LC159065–LC159071 (for 7 housekeeping genes).

**RESULTS**

**Isolation of a cdt-IB and cdt-IIIB gene-positive bacterium:** In the stool specimen containing isolate P2660, a 588-bp fragment corresponding to the cdtB gene was produced by PCR-RFLP (Fig. 1), which can detect and discriminate the cdtB genes of the 5 E. coli CDT strains (CDT-I to CDT-V) (12), and no other gastrointestinal pathogens (listed in the Materials and Methods section) were detected. Digestion of the PCR product with EcoRI/EcoRV yielded 4 fragments of 50, 130, 460, and 540 bp, whereas digestion with MspI yielded 5 fragments of 110, 160, 210, 320, and 360 bp. Thus, the total size of the digested fragments was 1,180 bp, which suggests that 2 different cdt genes might be present in this fragment. One appeared to be cdt-IB, and the other appeared to be cdt-IIIB (Fig. 1), suggesting the co-existence of a cdt-I gene-positive bacterium and a cdt-II gene-positive bacterium, respectively, or a single bacterium containing these 2 cdt genes. Subsequently, a single cdt gene-positive bacterium (P2660) was isolated and characterized. Isolate P2660 was negative for lactose/sucrose fermentation.
tation, citrate utilization, Voges-Proskauer reaction, and H2S and \( \beta \)-glucuronidase production, and positive for glucose fermentation and gas, indole, and lysine decarboxylase production, indicating that isolate P2660 was an atypical *E. coli*. The O serogroup of the isolate could not be determined since the somatic antigen of the isolate showed equal agglutination in the presence of O85 or O140 antiserum. PCR-RFLP analysis of the isolate showed an identical RFLP pattern, indicating the presence of 2 *cdt* genes in this bacterium.

**Virulence gene profiling:** To further characterize strain P2660, it was analyzed for the presence of virulence genes specific to diarrheagenic and necrotoxigenic *E. coli*. In addition to the 2 *cdt* genes, *eae* and *stx2* were detected by PCR (13). The *stx2* genes were subtyped by PCR and shown to be *stx2f* (16).

**Sequencing of *cdt-I*, *cdt-II*, and *stx2f***: Sequencing the flanking regions of the *cdt-IB*, *cdt-IIB*, and *stx2f* genes in strain P2660 yielded 2,383, 2,785, and 1,962 bp nucleotide sequences. The *cdt-I* gene cluster contained 3 adjacent ORFs, *cdt-IA* (714 bp/237 aa), *cdt-IB* (822 bp/273 aa), and *cdt-IC* (573 bp/190 aa). The *cdt-II* gene cluster also contained 3 ORFs, *cdt-IIA* (777 bp/258 aa), *cdt-IIB* (810 bp/269 aa), and *cdt-IIC* (546 bp/181 aa). The predicted amino acid sequences of the *cdt-I* genes were homologous to Cdt-IA (100% identity), Cdt-IB (99.6% identity), and Cdt-IC (100% identity) from *E. coli* strain 6468/62 (U03292), whereas the predicted sequences of the *cdt-II* genes were homologous to Cdt-IIA (99.2% identity), Cdt-IIB (98.5% identity), and Cdt-IIC (98.3% identity) of the prototype CDT-II strain (U04208). The amino acid residues important for DNaseI activity were conserved in both Cdt-IB (H153, G190-N193, D228, D259, and H260) and Cdt-IIB (H154, G191-N194, D229, D260, and H261). The *stx2f* gene cluster contained 2 ORFs, *stx2fA* (960 bp/319 aa) and *stx2fB* (264 bp/87 aa), which were nearly identical to the sequences of the *stx2f* genes (KF932377). These results indicated that the complete sequences of *cdt-I*, *cdt-II*, and *stx2f* were present in strain P2660.

![Fig. 2. Detection of CDT-I, CDT-II, and Stx2f expressed in the corresponding gene-positive bacterium strain P2660 by western blotting.](image1)

![Fig. 3. Cytotoxic effect of CDT-I, CDT-II, and Stx2f produced by the bacterium strain P2660.](image2)
Expression of CDT-I, CDT-II, and Stx2f: To confirm that strain P2660 produces CDT-I, CDT-II, and Stx2f, western blotting with anti-Cdt-IB, anti-PaCdtB, and anti-Stx2fA sera, respectively, was performed (Fig. 2). Bands corresponding to the sizes of Cdt-IB and Cdt-IIB in the positive controls were observed in the lysates of strain P2660, whereas no reactive bands corresponding to Stx2fA in the positive control were observed in the lysate of strain P2660 (Fig. 2). These results suggest that strain P2660 produced CDT-I and CDT-II. However, Stx2f production in strain P2660 could not be detected by western blotting.

Cytotoxicity of CDT-I, CDT-II, and Stx2f: To determine whether strain P2660 can produce biologically active CDT-I and CDT-II and whether Stx2f production can be detected by an alternative method, we used cytotoxicity assays. A filter-sterilized lysate of strain P2660 induced distention of CHO cells, which was also observed following the addition of CDT-I (Fig. 3A). Cell distention was still observed when CHO cells were intoxicated with a strain P2660 lysate that was pre-incubated with either anti-Cdt-IB or anti-PaCdtB antibodies, whereas little distention was observed with a strain P2660 lysate that was pre-incubated with both anti-Cdt-IB and anti-PaCdtB sera (Fig. 3A). These results indicated that strain P2660 produced biologically active CDT-I and CDT-II.

Strain P2660 lysate induced the death of Vero cells, which was also observed following the addition of Stx2a (Fig. 3B). Although Vero cell death was reduced in the presence of anti-Stx2fA serum, cell distention was observed. In contrast, cell death was observed in the presence of anti-Cdt-IB and anti-PaCdtB sera. However, no cytotoxic effects were observed in the presence of all 3 antisera (anti-Cdt-IB, anti-PaCdtB, and anti-Stx2fA; Fig. 3B). These results indicated that strain P2660 also produced biologically active Stx2f at a level that cannot be detected by western blotting.

Re-identification of strain P2660: Recently, it was reported that atypical EPEC might be misidentified as E. albertii (18). Therefore, to confirm the bacterial species of strain P2660, this bacterium was subjected to MLSA, the only currently available method that can clearly differentiate E. albertii from E. coli. The results showed that strain P2660 belonged to the E. albertii lineage (Fig. 4), indicating that strain P2660 is E. albertii but not E. coli.

DISCUSSION

E. albertii is a recently recognized human enteric pathogen (19,20). E. albertii is very similar to E. coli in terms of its biochemical properties, and it possesses a large chromosomal pathogenicity island, termed the locus of enterocyte effacement, which is involved in the formation of attaching and effacing lesions, similar to EPEC. Thus, several E. albertii strains have been misidentified as EPEC (18,21). In contrast to EPEC, a proportion of E. albertii strains harbor various toxin genes, including cdt (orthologs of E. coli cdt-I and cdt-II) and/or stx2 (stx2a and stx2f) (18,22). Since there are only a limited number of reports on the isolation and identification of E. albertii (18–24), more epidemiological information is needed to understand its pathogenicity and properties. In the present study, we identified a bacterium harboring cdt-I, cdt-II, and stx2f as E. albertii, which was isolated from a pediatric patient with diarrhea in Japan. The products of the 3 toxin genes were shown to be biologically active in mammalian cell lines, indicating that E. albertii strain P2660 may be highly virulent.

No well-known diarrheal pathogens which we examined were detected in the patient infected with E. albertii strain P2660, indicating that the strain might be the sole pathogen. In addition to the isolation of CDT-I-producing E. coli from diarrheal patients (25–27), Pandey et al. (28) reported an association between bloody diarrhea and high CDT-I production in EPEC, which causes watery diarrhea in infants. Moreover, a partially purified rCDT from S. dysenteriae, which is nearly identical to E. coli CDT-I, was shown to induce watery diarrhea in suckling mice (29). The significance of CDT-II production in the pathogenicity of E. coli and E. albertii is still not clear due to a lack of epidemiological data. However, bacteria producing CDT-II appear to be associated with diarrhea in humans since they have only been isolated from diarrheal patients and not from asymptomatic humans (Shima, unpublished). Therefore, strain P2660, which can produce both CDT-I and CDT-II, might be the etiological agent in this patient with diarrhea.

Although the Stx2fA toxin could not be detected by western blotting, the effects of toxin production could be detected by a cytotoxicity assay. This might be due
to the different sensitivities of the 2 methods. The cytoxicity assay (1.6 pg/well) (30) is more sensitive (~15,000 fold) than western blotting (25 ng/lane; data not shown). The significance of CDT and Stx production in the pathogenicity of \( E. albertii \) is not yet clear. However, the production of CDT-III or CDT-V but not CDT-I or CDT-II has been reported to be epidemiologically associated with disease in STEC infections (31). The CDTs identified in strain P2660 were CDT-I and CDT-II; however, the pathogenicity of the strain might depend on the production of CDT-I, CDT-II, and Stx2f as well as the intimin (an adhesin) encoded by \( eae \).

Although there is limited epidemiological information regarding the isolation of \( stx2f \) gene-positive \( E. albertii \) in human patients (18,23,24), it was recently reported that \( stx2f \) gene-positive \( E. coli \) accounted for 13% and 16% of all STEC clinical isolates in Belgium and the Netherlands, respectively (32,33), suggesting that \( stx2f \) gene-positive \( E. coli \) may be an emerging pathogen. Patients infected with \( stx2f \) gene-positive \( E. coli \) showed milder symptoms and a less severe clinical course than those infected with STEC serogroup O157 (33). Indeed, strain P2660 was isolated from a child with watery diarrhea, which is not as severe as the diseases caused by STEC O157 infection, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS). However, isolation of a \( stx2f \) gene-positive \( E. coli \) strain from a patient with HUS has been reported (34), suggesting an association between \( stx2f \) gene-positive \( E. coli \) and HUS. However, the presence of \( stx2f \) genes in \( E. coli \) and \( E. albertii \) isolates from both diarrheal patients and asymptomatic humans has been reported in Japan (18,23,24). In the present study, we show, for the first time, \( Stx2f \) production in an \( E. albertii \) isolate from a patient with diarrhea by using anti-\( Stx2f \)A-specific serum. Thus, our finding indicates that it is important to continuously survey diarrheal patients for \( E. albertii \) by molecular epidemiologic analysis and to identify emerging highly virulent \( E. albertii \) strains with various virulence genes.

In summary, an \( E. albertii \) strain was isolated as the sole pathogen from a child with diarrhea and was shown to harbor 3 different toxin genes, \( cdtr-I, cdtr-II \), and \( stx2f \), all of which showed biological activity. However, it is not clear how these 3 toxins are involved in the pathogenesis of the \( E. albertii \) strain. Additional studies are needed to evaluate the significance of the production of these 3 toxins by the \( E. albertii \) strain in the pathogenesis of diarrhea.

Acknowledgments This work was supported in part by Grant-in-aid for Scientific Research B (KAKENHI) from JSPS.

Conflict of interest None to declare.

REFERENCES

E. albertii Produces CDT-I, CDT-II, and Stx2f