Cloning and Characterization of Genes Encoding Homologues of the B Subunit of Cholera Toxin and the Escherichia coli Heat-Labile Enterotoxin from Clinical Isolates of Citrobacter freundii and E. coli

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We identified and characterized a gene encoding a homologue of the B subunits of cholera toxin (CTB) and heat-labile enterotoxin (LTB) of Escherichia coli from a clinical isolate of Citrobacter freundii that was found to produce a factor in the culture supernatant that cross-reacted with antibodies to CTB and LTB when assayed by enzyme-linked immunosorbent assay (ELISA). The gene encoding the ELISA-positive factor, cfxB, consisted of 375 nucleotides and was located downstream of an 852-nucleotide open reading frame, cfxA, with a 56-nucleotide intergenic space. The cfxB gene was predicted to encode a 125-amino-acid polypeptide, which had 73.8 and 72.8% identities with the amino acid sequences of LTB and CTB, respectively. However, the amino acid sequence of the deduced polypeptide CFXA had no homologies to those of the A subunits of CT or LT. DNA probes developed from the sequences of cfxA and cfxB were used to screen 67 C. freundii isolates and 152 E. coli isolates from diarrheal patients by colony blot hybridization. Two strains, C. freundii 48 and E. coli 176, reacted with both DNA probes under conditions of high stringency. We cloned homologues of the cfxA and cfxB genes from E. coli 176 and designated them ecxA and ecxB, respectively. The ecxA gene and the ecxB gene comprise 855 and 375 nucleotides, respectively, with a 50-nucleotide intergenic space, and encode a 285- and a 125-amino-acid residue polypeptides, respectively. The results of the present study may provide important clues to the origin and evolution of immunologically related factors sharing a common enterotoxin-like A and B subunit structures.

Enteric bacterial pathogens including Vibrio cholerae and enterotoxigenic Escherichia coli often produce ADP-ribosylating enterotoxins that are mainly responsible for the diarrheal disease caused by these bacteria. Two well-characterized enterotoxins include cholera toxin (CT) produced by toxigenic strains of V. cholerae and the heat-labile toxin (LT) produced by enterotoxigenic E. coli (ETEC) strains. The origin and evolution of genes encoding these toxins, which typically consist of a catalytic A subunit and a receptor-binding and immunogenic B subunit, have not been adequately explored. Citrobacter freundii is a member of the Enterobacteriaceae family and is commonly found in the environment, as well as in the intestinal tracts of both animals and human. This ubiquitous organism can also cause gastroenteritis in human (4, 12). Previous studies have detected the production of LT- or CT-like factors by C. freundii by using immunological methods and PCR (2, 9). In the present study, we describe molecular cloning and characterization of such LT- and/or CT-like factor produced by a clinical isolate of C. freundii 09-1.

The C. freundii strain was isolated from a child with diarrhea in a survey of diarrheagenic bacteria in 0- to 3-year-old children in Lecife, Brazil. The identity of the strain was confirmed by using the bacterial identification test kits EB-20 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), BioTest No. 1 (Eiken Chemical Co., Ltd., Tokyo, Japan), and API 20NE (Bi-oMérieux-Japan, Ltd., Tokyo, Japan). Culture supernatants of the strain were also tested for immunological cross-reactivity with anti-CT polyclonal antibody with standard kits for detection of LT and CT. This included a passive latex agglutination kit (VET-RPLA; Denka Seiken Co., Ltd., Tokyo, Japan), and a bead enzyme-linked immunosorbent assay (ELISA) kit (BT Test Nissui CT; Nissui). To determine whether culture supernatants of different strains had any biologically toxic activity, we tested the supernatant by using the Chinese hamster ovary (CHO) cell assay and the suckling mouse assay as described previously (1, 8).

C. freundii 09-1 was tested for the presence of genes homol-
ogous to the genes for the A and B subunits of CT and LT (CTA and CTB and LTA and LTB, respectively) by using specific DNA probes (15). These probes included a 0.68-kb XbaI-EcoRI fragment of the gene encoding LTA, a 0.24-kb EcoRI-HinclII fragment of the gene for LTB, and a 0.54-kb XbaI-ClaI fragment of the gene for CTA (10, 13, 15). Relevant fragments of DNA were cloned from strain 09-1 by constructing a genomic library and screening by immunological methods. Briefly, DNA libraries were constructed by ligating endonuclease-digested total DNA from the strain 09-1 with pUC119 and transforming *E. coli* MC1061. The transformants were screened by using the BT Test Nissui CT bead ELISA kit. The nucleotide sequencing of relevant clones was performed by using a 7-DEAZA sequencing kit (Takara Shuzo Co., Kyoto, Japan) in accordance with the manufacturer's instructions. The nucleotide sequences obtained for *cfxAB* and *ecxAB*, respectively, were deposited in GenBank under accession numbers AB076667 and AB076668.

*C. freundii* strain 09-1 presented a weakly positive result in the reversed passive latex agglutination kit for LT and CT detection, which uses the anti-CT polyclonal antibody. This result was also confirmed by a bead ELISA with a different anti-CT polyclonal antibody. This strain, however, did not react with DNA probes for the A or B subunits of CT and LT in colony blot hybridization tests under conditions of high stringency (10). However, screening of a genomic library derived from strain 09-1 with the anti-CT antibody identified a positive clone carrying a 4.2-kb *Pst*I fragment of the *C. freundii* genome. This DNA insert was subjected to deletion analysis to identify the region responsible for the positive immunoassay. Deletion analysis showed that the genomic region encoding the ELISA positive factor was located on a 1.5-kb *Xba*I-*Bam*HI fragment (Fig. 1). A 375-nucleotide open reading frame (ORF) was identified, which we designated *cfxB* (Fig. 2).

The *cfxB* gene encoded a 125-amino-acid polypeptide and ex-

FIG. 1. Restriction map of genes encoding CFX, LT, and CT.

FIG. 2. Alignment of amino acid sequences of CFX, ECX, LT, and CT. CLUSTALW version 1.8 was used to align the sequences. Identical amino acid residues are indicated by an asterisk, and conserved substitutions are indicated by colons (= strongly conserved) and periods (= weakly conserved). LTB and CTB data are from DDBJ/EMBL/GenBank under accession numbers J01646 and X58785, respectively.
hhibited 73.8 and 72.8% identities in amino acid sequences compared to that of LTB and CTB, respectively (Table 1). This indicated that the product of cfxB gene (CFXB) was most likely the immunologically cross-reacting LT/CT-like factor. CFXB has a highly conserved immunogenic region of serine-55-isoleucine-58 (11) and of threonine-47-lysine-68 (6) as in LTB and CTB (Fig. 2). Enterotoxins such as CT and LT consist of an enzymatic A subunit and a B subunit that bind to appropriate receptors. In the present study, nucleotide sequencing identified an 852-nucleotide ORF upstream of the cfxB gene with a 56-nucleotide intergenic space. Considering the analogy with genes encoding CT and LT, we designated this 852-base ORF as the cfxA gene. The predicted amino acid sequence of CFXA, however, did not have homologies to that of LTA or CTA.

We studied the distribution of the cfxA and cfxB genes among 67 C. freundii isolates and 152 E. coli isolates from diarrheal patients in Japan by colony hybridization tests with the 0.85-kb PshHI fragment of the cfxA gene and the 0.3-kb HindIII fragment of the cfxB gene (Fig. 1). Two strains, C. freundii 48 and E. coli 176, reacted with both DNA probes for cfxA and cfxB under conditions of high stringency. Subsequently, we cloned homologues of cfxA and cfxB genes from E. coli 176 and designated them ecxA and ecxB, respectively. Nucleotide sequencing showed that the ecxA gene and ecxB genes comprise 855 and 375 nucleotides and encoded 285- and 125-amino-acid polypeptides, respectively. As in the cfxAB genes, a 50-nucleotide intergenic space was found between ecxA and ecxB. The deduced amino acid sequence of the cfxA and ecxA gene products had a 87.4% identity. Comparisons of amino acid sequences of the B subunits of CFX, ECX, LT, and CT (CFXB, ECXB, LTB, and CTB, respectively) are given in Table 1. When culture supernatants of strains C. freundii 09-1 and C. freundii 48 and E. coli 176 were tested for biological toxicity, the culture supernatants did not cause CHO cell elongation or fluid accumulation in the suckling mouse assay. This result suggested that, although the factors produced by the C. freundii strains were immunologically cross-reactive to CTB and LTB, there was no biological activity, which is normally expressed by the A subunit of LT or CT.

Recent molecular studies on V. cholerae revealed that virulence genes are dispersed among environmental strains of V. cholerae and may be carried about on mobile genetic elements (3, 5), thus enabling V. cholerae to receive or transfer genes leading to new pathogenic variants (3). Also, it should be added that certain strains of C. freundii have been reported to produce E. coli heat-stable enterotoxin (7) or Shiga-like toxin (14). This suggests a possible gene transfer between C. freundii and E. coli. However, in the present study, although the factor produced by the C. freundii strains cross-reacts immunologically with LT and CT, there was no evidence for biological activity. Moreover, there was no considerable homology in the nucleotide sequence of the cfxAB and cfxAB genes, suggesting the evolution of immunologically related factors sharing common enterotoxin-like A and B subunit structures in different species of bacteria. In the present study we characterized two C. freundii isolates and one E. coli isolate that carried the cfxAB and ecxAB genes, respectively. Further studies are required to understand the significance and distribution of these genes in other bacterial species.

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REFERENCES