INFECTION AND IMMUNITY, Mar. 1991, p. 1065–1073
0019-9567/91/031065-09$02.00/0

Vol. 59, No. 3

Two Copies of Shiga-Like Toxin II-Related Genes Common in Enterohemorrhagic *Escherichia coli* Strains Are Responsible for the Antigenic Heterogeneity of the O157:H$^-$ Strain E32511

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Received 19 September 1990/Accepted 3 January 1991

Thirty-two clinical isolates of Shiga-like toxin (SLT)-producing *Escherichia coli* associated with single cases or outbreaks of bloody diarrhea, hemorrhagic colitis, the hemolytic uremic syndrome, or edema disease of swine were examined for multiple copies of genes belonging to the *slt*-I or *slt*-II toxin families. Five of 19 strains that were known to produce SLT-II or to hybridize to *slt*-II-specific probes by colony blot were found by Southern hybridization to contain two copies of toxin genes related to *slt*-II. The genes for two toxins closely related to *slt*-II were cloned from one of the isolates, *Escherichia coli* O157:H$^-$ strain E32511. One copy of the operon was found to be essentially identical to *slt*-II; it differed from *slt*-II by only one nucleotide base. This single nucleotide difference did not affect the predicted amino acid sequence. The predicted amino acid sequence of the A subunit of the second operon was identical to that of SLT-II, but the predicted amino acid sequence of the B subunit was identical to that of the B2F1 toxin VT2ha. We designated this second operon *slt*-IIc. Neutralization assays using several monoclonal antibodies and polyclonal antiserum prepared against SLT-II showed that SLT-IIc was antigenically related to but distinct from SLT-II.

Several studies have shown that *Escherichia coli* strains of serotypes O157:H7, O157:H$^-$, O26:H11 and others are associated with hemorrhagic colitis (10a, 29b, 34) and hemolytic-uremic syndrome (HUS) (11a, 38a, 44). These strains produce Shiga-like toxins (SLT), also called Vero toxins (VT) (14), that are cytotoxic for both Vero cells and HeLa cells. There are two antigenically distinct groups of SLT: SLT-I and SLT-II (for reviews, see references 11, 27, and 39). Members of the SLT-I group appear to be antigenically homogeneous, but evidence of a diverse but related family of SLT-II toxins is growing. The nucleotide sequences of two toxin operons closely related to SLT-II and found in an *E. coli* O91:H21 strain isolated from a patient with HUS were recently presented (9). This characterization of the *vtx*2ha and *vtx*2hb genes from strain B2F1 was the first demonstration of multiple copies of toxin genes related to SLT-II present within a single strain (9).

It has been proposed that the *E. coli* O157:H$^-$ strain E32511 produces yet another distinct toxin in the SLT-II (VT2) family (5a). Two variants of SLT-II have been well characterized (4, 46). The variants, designated SLT-IIv and SLT-IIva, unlike SLT-II, are cytotoxic for Vero but not HeLa cells. The difference in cytotoxicity for Vero and HeLa cells between SLT-II and SLT-IIv may be due to different receptor specificities (35). SLT-IIv is the causative agent of edema disease in pigs (15, 22), while SLT-IIva has been associated with diarrhea in a human infant (4).

Toxins produced by *E. coli* 933 and E32511 have been studied for production of SLT-II and VT2, respectively (37a, 41). The terms SLT and VT are used interchangeably to describe these toxins; however, it is not clear whether SLT-II and VT2 are identical toxins. Both toxins are found on and have been cloned from bacteriophages (25, 38a, 42, 48). The sequence of the *slt*-II operon from 933 is known (10). However, reports on the capacities of various anti-SLT (VT) sera to neutralize toxin preparations from 933 and E32511 have been somewhat contradictory. Two groups concluded that SLT-II of 933 and VT2 of strain E32511 are similar or identical by serological criteria (41, 49), while two other groups indicated that SLT-II from strain 933 and VT2 from strain E32511 are antigenically distinct but related (2, 5a). In this study, 32 clinical isolates were examined to determine the prevalence of multiple copies of similar toxin genes. In addition, we report the cloning, sequencing, and antigenic characterization of two toxin operons from *E. coli* O157:H$^-$ strain E32511. The nucleotide sequences of the toxin genes were compared with those of other toxin genes in the SLT-II family.

MATERIALS AND METHODS

Plasmids, phages, and strains. Plasmids, phages, and bacterial strains used in this study are listed and described in Tables 1 and 3.

Media, enzymes, biochemicals, and radionuclides. Strains were routinely grown in L broth (24) for 18 h at 37°C. When necessary, antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to the medium to the following final concentrations: ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 12.5 μg/ml. Media were also supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co.) as needed. Restriction endonucleases and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim, Indianapolis, Ind., and T4 DNA ligase was obtained from Boehringer Mannhiem or U.S. Biochemicals Corporation, Cleveland, Ohio. Sequenase was purchased from U.S. Biochemicals, and Gigapack II Gold Packaging Extract was from Stratagene, La Jolla, Calif. Enzymes were used according to the instructions of the suppliers. Radionuclides were

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TABLE 1. Plasmids, phages, and strains used in this study

Plasmid, phage, or strain	Characteristic(s)	Source or reference[a]
Plasmids		
pHC79	Cosmid vector; Ap^r, Tc^r	7
pCKS25	pHC79 clone of E32511; Ap^r	This study
pCKS37	pHC79 clone of E32511; Ap^r	This study
pRK415	Low copy vector; Tc^r	12
pCKS109	pRK415 subclone from pCKS25; SLT-II$^+$; Tc^r	This study
pCKS111	pRK415 subclone from pCKS37; SLT-IIc$^+$; Tc^r	This study
pLP32	SLT-II$^+$	35
pJES101	SLT-IIv$^+$	35
Phages		
M13mp18 and mp19	Sequencing vectors	26
E. coli strains		
XL1-Blue	Host for M13 phage; Tc^r	Stratagene
DH5α	Host strain for cloning	BRL
E32511	Clinical isolate of *E. coli* associated with HUS	CDC
C600(933W)	C600 lysogenized with phage 933W; SLT-II$^+$	H. W. Smith
S. dysenteriae type 1 60R	Rough; noninvasive; Shiga toxin$^+$	S. Formal

[a] BRL, Bethesda Research Laboratories; CDC, Centers for Disease Control.

purchased from New England Nuclear Research Products, Boston, Mass.

DNA manipulations. Large-scale preparation of total DNA was by the method of Marmur (20). Small-scale preparation of total DNA from 1.5-ml cultures of clinical isolates was performed as described in *Current Protocols in Molecular Biology* (1). Plasmid DNA was isolated by the method of Holmes and Quigley (8). Host cells were made competent to take up DNA by calcium chloride and heat shock (17). Procedures for cosmid cloning and subcloning were those of Maniatis et al. (18). Cosmids were packaged by using the Stratagene Gigapack II Gold Packaging Extract.

DNA hybridization studies. Chromosomal and plasmid DNAs were probed with *slt*-specific sequences by in situ gel hybridization (12a) with the following modifications. Gels were not prehybridized, and 20 mM sodium pyrophosphate was included in the hybridization solution as a blocking agent. Washing conditions were as follows. One wash was done in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) at 65°C for 1 h, followed by one wash in 0.1× SSC at 65°C for 1 h. DNA probes specific for *slt*-I and *slt*-II were labeled with [^{32}P]dCTP by nick translation (19). Clones containing complete or partial toxin genes were identified by colony or plaque hybridization (18).

Nucleotide sequence analysis. DNA sequencing followed the dideoxy-chain termination method of Sanger et al. (36) as adapted for M13 vectors by Schreier and Cortese (37). Specifically, the Sequenase kit from U.S. Biochemicals was used for nucleotide sequencing. M13mp18 and mp19 vectors were used for cloning and sequencing as described by Messing and coworkers (26). In addition to the M13 universal sequencing primer, several *slt*-II-specific synthetic oligonucleotides (Applied Biosystems Incorporated model 380A synthesizer) (10) were used in sequencing.

Cytotoxicity assays. Cytotoxicity assays were done essentially as described by Gentry and Dalrymple (5). Wild-type *E. coli* and various *E. coli* clones and subclones were cultured as described above and disrupted by sonic lysis as described previously (28). The filter-sterilized bacterial lysates were serially diluted in tissue culture medium (Dulbecco modified Eagle medium containing 10% fetal calf serum, 0.8 mM glutamine, 500 U of penicillin G per ml, and 500 mg of streptomycin per ml). One hundred microliters of 10-fold dilutions of the bacterial lysate was added to individual wells of a 96-well microtiter plate which contained approximately 10^4 Vero or HeLa cells in 100 μl of medium. The tissue culture cells were then incubated at 37°C in the presence of 5% CO_2 for 24 (HeLa cells) or 48 (Vero cells) h and then fixed and stained with crystal violet (16). The intensity of color of the fixed and stained cells was measured with a Titertek Multiskan MC reader at 620 nm (Flow Laboratories, Inc., McLean, Va.). Fixed and stained toxin-treated tissue culture cells were compared with fixed and stained untreated cells; stain intensity was proportional to the number of viable, attached tissue culture cells present before being fixed to the well. The 50% cytotoxic dose represented the amount of toxin required to kill 50% of the cells in a well.

Neutralization of cytotoxicity. The antibodies used are listed and described in Table 2. All monoclonal antibodies

TABLE 2. Antibodies used in this study

MAb or rabbit antitoxin[a]	Specificity[b]	Isotype[c]	Source or reference
MAbs			
11G10	SLT-II A subunit	IgM(κ)	32
2E1	SLT-II A subunit	IgM(κ)	32
BC5 BB12	SLT-II B subunit	IgG(κ)	3
13C4	SLT-I B subunit	IgG	41
Sera			
AJ65	SLT-II	Polyclonal	42
F45	Shiga toxin	Polyclonal	29
AD13	Normal rabbit serum	Polyclonal	This lab

[a] All monoclonal antibodies (MAbs) and sera were prepared in this laboratory, except BC5 BB12, which was kindly supplied by N. A. Strockbine.

[b] Shiga toxin and SLT-I are essentially identical and are immunologically cross-reactive (40) (Fig. 4).

[c] IgM, immunoglobulin M.

and sera were prepared in this laboratory except BC5 BB12, which was kindly supplied by Nancy Strockbine, Centers for Disease Control, Atlanta, Ga. The F45 antitoxin was prepared against purified Shiga toxin from *Shigella dysenteriae* type 1 60R; Shiga toxin and SLT-I are essentially the same (40). AJ65 antitoxin was prepared by immunizing rabbits with crude preparations of sonically disrupted *E. coli* C600(933W), an SLT-II-producing lysogen. Antibodies BC5 BB12 (ascites fluid) and AJ65 (antiserum) were diluted 10-fold to prepare starting stocks. Starting stocks of hybridoma supernatants (11G10 and 2E1) were not diluted, nor was antiserum F45.

Monoclonal antibodies and polyclonal sera to be tested for cytotoxin-neutralizing activity were serially diluted in tissue culture medium. A sample (100 μl) of diluted toxin (containing approximately 40 times the 50% cytotoxic dose) was added to an equal volume of each antibody dilution and incubated at 37°C for 2 h, followed by incubation at 4°C for 4 h. A sample (100 μl) of each mixture was then transferred to Vero cells, and the microtiter plates were incubated and tissue culture cells were fixed and stained as described above. Toxin in the absence of antibody killed between 60 and 90% of the tissue culture cells in a well, as determined below. Percent neutralization was determined by the following formula: $\{[A_{620}(\text{toxin} + \text{antibody}) - A_{620}(\text{toxin})]/A_{620}(\text{untreated cells})\} \times 100$.

The experiments reported here were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (24a).

Nucleotide sequence accession number. The nucleotide sequence of *slt*-IIc has been submitted to EMBL and assigned the accession number M59432.

RESULTS

Multiple toxin genes in clinical isolates. To determine the prevalence of multiple copies of toxin genes in clinical isolates, 32 clinical isolates were chosen to be probed with *slt*-I- and/or *slt*-II-specific DNA sequences. Earlier studies have shown that 7 of the 32 isolates expressed SLT-I, 1 expressed SLT-IIv, 19 expressed SLT-II, and 5 expressed both SLT-I and SLT-II (21). Total DNA was isolated, digested with *Eco*RI, and subjected to electrophoresis through a 0.9% agarose gel. These gels were then probed with toxin-specific probes by in situ gel hybridization. The *slt*-I probe used was a 913-bp *Hin*cII fragment isolated from a partial digest of the plasmid pNAS13 (40), which encodes *slt*-I; this *Hin*cII fragment spanned the A and B subunit coding regions. The *slt*-II probe used was the 841-bp *Sma*I-*Pst*I fragment of pLP32 (35), which contains only A subunit gene sequences. Figure 1 shows an example of one gel probed with the *slt*-IIA sequences. The SLT-I-expressing strain H19 was included to show the specificity of the *slt*-IIA probe (Fig. 1, lane 2). Table 3 summarizes results obtained from probing restricted DNA of the 32 clinical isolates with *slt*-specific fragments. Five strains were identified with two *Eco*RI fragments that hybridized with the *slt*-IIA probe (Fig. 1; Table 3). In no case were multiple fragments found to hybridize with the *slt*-I probe.

Cloning of toxin genes from *E. coli* E32511. Total DNAs from *E. coli* E32511 and C600(933W) were digested with several restriction endonucleases and subjected to electrophoresis through a 0.9% agarose gel. This gel was then probed with the 841-bp *Sma*I-*Pst*I fragment of pLP32 to determine the sizes of fragments hybridizing with the A subunit of SLT-II (*slt*-IIA). Each restriction enzyme used to digest total DNA from the control strain C600(933W) generated a unique fragment that hybridized with the probe (data not shown). However, multiple bands hybridized when E32511 DNA was probed (data not shown), suggesting that more than one copy of a toxin gene may be present. DNA from E32511 digested with *Eco*RI gave two bands of approximately 4.8 and 5.5 kb which showed hybridization with the *slt*-IIA probe (Fig. 1, lane 7).

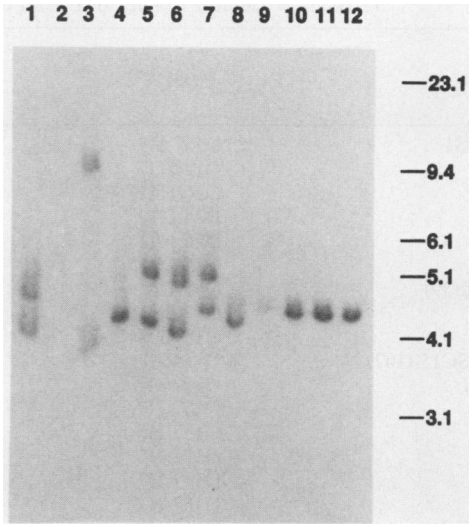

FIG. 1. In situ gel hybridization of DNA from clinical isolates. Total DNAs were digested with *Eco*RI and hybridized with the 841-bp *Sma*I-*Pst*I *slt*-IIA probe. The clinical isolates from which DNAs were obtained were J-2 (lane 1), H19 (lane 2), B2F1 (lane 3), B2387 (lane 4), B1409-C1 (127) (lane 5), B1375-GSC15 (107) (lane 6), E32511 (lane 7), CL8 (lane 8), A8959-C7 (lane 9), A9047-CS1 (lane 10), EDL933 (lane 11), and CL40 (lane 12). Numbers to the right are marker DNA fragments in kilobase pairs.

To characterize the toxin(s) of E32511, a cosmid bank of a *Sau*3A partial digest was created in the cosmid vector pHC79. Cosmids were initially screened by colony hybridization with the *slt*-IIA probe and then by in situ gel hybridization using *Eco*RI-digested cosmid DNA. Several cosmids containing either a 4.8- or a 5.5-kb *Eco*RI fragment that showed hybridization with the *slt*-IIA probe were obtained; the two fragments were never found in a single cosmid clone. Representative cosmids containing each fragment were purified for further analysis. Bacterial lysates of *E. coli* DH5α with cosmids that contained either fragment were cytotoxic for Vero cells, which indicated that an entire toxin operon was present on each clone (data not shown). The two *Eco*RI fragments of interest were subcloned from the cosmids into the vector pRK415, which yielded the plasmids pCKS109, with the 4.8-kb *Eco*RI fragment, and pCKS111, with the 5.5-kb *Eco*RI fragment.

Sequencing of toxin genes. Portions of the toxin genes of E32511 were obtained by isolating the 4.8- or 5.5-kb fragment either from the parent cosmids or from the pRK415 subclones and digesting these with *Hae*III, *Hin*cII, or *Pst*I. The products of these restriction digests were then ligated into appropriate M13 vectors; digestion with *Hae*III or *Hin*cII and subsequent ligation generated a mixture of clones. Clones were selected for sequencing following hybridization with probes derived from pLP32, which covered the *slt*-II coding region. The nucleotide sequence of approximately 1.5 kb of DNA spanning each of the toxin operons

TABLE 3. Clinical isolates of *E. coli* that contain DNA hybridizing with *slt*-I and *slt*-II probes or both

Strain	Serotype[a]	SLT type[b]	Source or reference[c]	No. of probe-positive fragments[d]	
				slt-I	*slt*-II
B6550 MS1	O157:H7	I	CDC	1	ND
A9167-1	O157:H7	I	CDC	1	ND
H19	O26:H11	I	H. R. Smith	1	0
1557-77	O26:H11	I	M. M. Levine	1	ND
CL5	O26:H11	I	M. A. Karmali	1	ND
D276/1/1	O111:NM	I	D. Sherwood	1	ND
A9619-C2(29)	O45:H2	I	CDC	1	ND
S1191	O139:ND	IIv	S. Cryz	ND	1
E32511	O157:NM	II	CDC	ND	2
B1375-GSC15 (107)	O157:H7	II	CDC	ND	2
B1409-C1	O157:H7	II	CDC	ND	2
B1545	O157:H7	II	CDC	ND	1
B2387	O157:H7	II	CDC	ND	1
B2576	O157:H7	II	CDC	ND	1
001	O157:H7	II	Dr. Dudevoir	ND	1
B5329	O157:H7	II	CDC	ND	1
B5311-C1	O157:H7	II	CDC	ND	1
J-2	O157:H7	II	Y. Takeda	ND	2
86-24	O157:H7	II	P. I. Tarr	ND	1
B6181 MS2-1	O157:H7	II	CDC	ND	1
B6270 MS1-1	O157:H7	II	CDC	ND	1
B6226 MS1-2	O157:H7	II	CDC	ND	1
B6612 MS1	O157:H7	II	CDC	ND	1
B2619/2/1	O8:H9	II	D. Sherwood	ND	1
B1696/2/1	O4:NM	II	D. Sherwood	ND	1
B2F1	O91:H21	II	M. A. Karmali	ND	2
B6232 MS1	ND:NM	II	CDC	ND	1
EDL933	O157:H7	I+II	CDC	1	1
CL40	O157:H7	I+II	M. A. Karmali	1	1
A9047-CS1	O157:H7	I+II	CDC	1	1
A8959-C7	O157:H7	I+II	CDC	1	1
CL8	O157:H7	I+II	M. A. Karmali	1	1

[a] ND, Not determined.
[b] SLT type was determined by bioassay and/or colony blot data (21).
[c] CDC, Centers for Disease Control.
[d] Hybridization with *slt*-I- or *slt*-II-specific probes was determined by using *Eco*RI-digested chromosomal DNA. The number of unique fragments hybridizing with the probe(s) is presented.

was determined for both strands, except that the nucleotide sequence of only one strand was determined for a small portion of the extreme 5′ end of each A subunit gene.

Because we had difficulty aligning the new toxin sequences with that of *slt*-II, the region of *slt*-II from pLP32 spanning positions 392 to 403 was resequenced. The plasmid pLP32 was derived from pNN76; the DNA sequence of the *slt*-II operon was originally determined from pNN76 (10). This region of *slt*-II was found to contain three additional nucleotide bases not present in the previously published sequence (10). The revised sequence is presented here. These alterations result in the change of histidine (+45) to proline (+45) and the insertion of an additional proline (+46).

Figure 2 shows a comparison of the sequences of *slt*-II (top line) and the toxin genes from pCKS111. Several base differences were found upstream of the promoter region and between the −10 region and the Shine-Dalgarno sequence of the A subunit gene. The first base of the −35 region, position 44, was a cytosine in *slt*-II and an adenine in the toxin gene from pCKS111. Four base differences were found within the structural gene for the A subunit; none of these affected the amino acid sequence. However, 11 differences were found in the B subunit gene, which resulted in three amino acid substitutions (Fig. 3); two of these were within the mature B subunit. For reasons to be discussed below, this operon was designated *slt*-IIc. The sequence of the toxin gene from pCKS109 differed from that of *slt*-II in only one base; the guanine at position 325 was replaced by an adenine (data not shown). This substitution does not affect the amino acid sequence of the toxin; therefore, this operon was referred to as *slt*-II.

The nucleotide and predicted amino acid sequences of the A and B subunits of *slt*-IIc were compared with those of others in the SLT-II family (Table 4). The *slt*-IIc A subunit was found to be most similar to that of *slt*-II at the nucleotide level and was identical to SLT-IIA at the protein level. However, the *slt*-IIc B subunit is identical to the B subunit of *vtx*2ha at both the nucleotide and protein level (Table 4; Fig. 3). Thus, in keeping with both the Shiga-like toxin-II (10) and the *vtx*2ha/*vtx*2hb (9) nomenclatures, we designated this operon *slt*-IIc.

Cytotoxicity and neutralization of toxins. The toxin subclones were tested for cytotoxicity for Vero and HeLa cells. Controls included were Shiga toxin, which is essentially the same as SLT-I (40), SLT-II, and SLT-IIv toxins. Toxins from the parent, E32511, and *E. coli* DH5α(pCKS109) gave comparable 50% cytotoxic doses per milliliter on both cell types, as did the controls SLT-II and Shiga toxin (Table 5). However, toxin from *E. coli* DH5α(pCKS111) was about

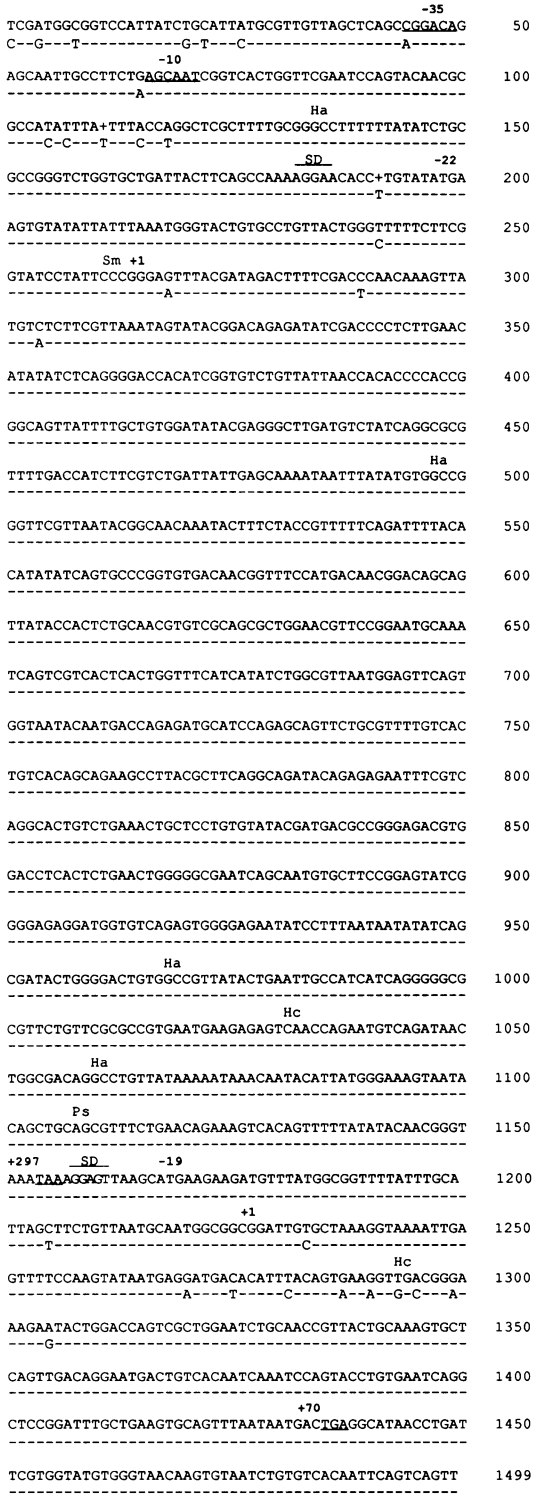

FIG. 2. Comparison of the nucleotide sequences of *slt*-II and the toxin genes from pCKS111. DNA sequence extends from 43 bp 5′ of the promoter region of *slt*-II to 62 bp 3′ of the B subunit termination codon. The sequence and putative regulatory elements of *slt*-II, as determined by others (10), are presented in the top line. The sequence of the toxin from pCKS111 is shown below. Hyphens (-) indicate nucleotide identity; plus signs (+) represent nucleotide absence. Putative regulatory elements presented are transcriptional initiation signals (−35 and −10 regions) and Shine-Dalgarno sequences (SD). The initiator codons of the A and B subunits are designated −22 and −19, respectively, and the amino-terminal residues of the processed A and B subunits are designated +1. Restriction sites relevant to subcloning and *slt*-II-specific probe generation are as follows: Ha, *Hae*III; Hc, *Hinc*II; Ps, *Pst*I; Sm, *Sma*I.

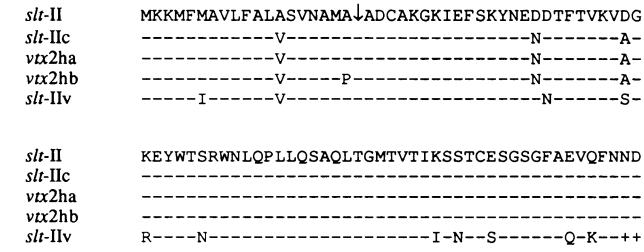

FIG. 3. Comparison of the amino acid sequences of the B subunits of the *slt*-II, *slt*-IIc, *vtx*2ha, *vtx*2hb, and *slt*-IIv operons. Hyphens (-) indicate residues identical to *slt*-II. Plus signs (+) indicate the absence of a residue. The arrow (↓) shows the signal sequence cleavage junction. Deduced amino acid sequences other than that for SLT-IIc are from published reports (9, 10, 46).

10-fold less cytotoxic for HeLa cells than for Vero cells, while SLT-IIv was about 10,000-fold less toxic for HeLa cells than for Vero cells.

Several antibodies were tested for neutralizing capacity against these toxins. Neutralization of sonically disrupted cultures of wild-type E32511 (contains *slt*-II and *slt*-IIc genes) and *E. coli* DH5α(pCKS109) (expresses SLT-II cloned from E32511) by the monoclonal antibodies specific for the A (11G10, 2E1) or B (BC5 BB12) subunit of SLT-II was comparable to that of the SLT-II control (Fig. 4A, B, and C). All three anti-SLT-II monoclonal antibodies were able to neutralize the activity of the toxins produced by strain DH5α(pCKS111) (expresses SLT-IIc), but at a much reduced titer, compared with that of SLT-II toxin. This panel of monoclonal antibodies did, however, neutralize SLT-IIc toxin more efficiently than SLT-IIv toxin or Shiga toxin. Polyclonal antiserum prepared against SLT-II (AJ65) neutralized the toxicity of bacterial lysates of clones expressing SLT-IIc and SLT-IIv toxins less efficiently than it did those expressing SLT-II toxin or bacterial lysates of strain E32511 (Fig. 4D). Neither a monoclonal antibody specific for the B subunit of SLT-I toxin (13C4) nor polyclonal antiserum prepared against Shiga toxin (F45) neutralized the toxicity of lysates of strains or clones that expressed any member of the SLT-II family (data not shown). Furthermore, normal rabbit serum had no effect on cytotoxicity of any of the bacterial lysates (data not shown).

DISCUSSION

In this study, 26% of the SLT-II-expressing enterohemorrhagic *E. coli* (EHEC) isolates examined contained two *Eco*RI chromosomal fragments that hybridized to *slt*-II-specific DNA probes. Because none of the *slt* operons sequenced to date have contained an *Eco*RI site within or between the structural genes for the A and B polypeptides, we concluded that any EHEC strain with two *slt*-II-hybridizing *Eco*RI fragments has two copies of *slt*-II-related genes. Whether each copy encodes a functional toxin was not determined, except for strain E32511. When the presence of two (or more) copies of related but distinct toxin genes in a

TABLE 4. Comparison of the nucleotide and predicted amino acid sequences of the processed A and B subunits of the *slt*-IIc genes with those of the SLT-II family

Operon[a]	% Homology of[b]:			
	Nucleotide sequence		Predicted amino acid sequence	
	A subunit	B subunit	A subunit	B subunit
slt-IIc	100 (100)[b]	100 (100)	100 (100)	100 (100)
*vtx*2ha	98.5 (98.5)	100 (100)	99.0 (99.1)	100 (100)
*vtx*2hb	98.7 (98.6)	98.6 (98.5)	99.0 (99.1)	100 (98.9)
slt-II	99.7 (99.6)	95.2 (94.8)	100 (100)	97.1 (96.6)
slt-IIv	94.6 (94.4)	79.0 (82.8)	94.2 (94.2)	82.9 (85.4)

[a] Sequences for *vtx*2ha and *vtx*2hb, *slt*-II, and *slt*-IIv were from references 9, 10, and 46, respectively.
[b] Values represent percent homology with the indicated sequences from the A and B subunits of *slt*-IIc. Numbers in parentheses are homologies comparing unprocessed gene products.

clinical isolate goes unrecognized, any results obtained on the genetic and immunological characteristics of that strain may be erroneously interpreted. Furthermore, any toxin isolated to homogeneity from such a strain is potentially impure. Thus, the toxin prepared by Head et al. (6) from strain E32511 probably consists of the two comparable but not identical toxins, SLT-II and SLT-IIc. Similarly, the VT2 purified from EHEC B2F1 (30) is probably also impure because Ito et al. recently reported that this strain contains two closely related but distinct SLT-II-like toxins (9). Furthermore, the VT2 isolated from EHEC strain J-2 (49) may also contain two populations of similar but not absolutely homologous toxins because our results suggest the possibility of two *slt*-II genes in that strain (Fig. 1). Finally, VT2 has also been purified from strain E3657 (2), and it would be prudent to analyze this strain for additional copies of *slt*-II. The issue of two different types of SLTs in a preparation of purified toxin arose in this laboratory when we became aware through the finding of Scotland et al. (37a) that EHEC 933 expresses both SLT-I and SLT-II. The fact that the SLT-I we isolated from strain 933 was apparently pure (29a) was solely due to the facts that we used an antitoxin affinity column in our purification scheme and SLT-I and SLT-II are not immunologically cross-reactive (10) (Fig. 4). However, the possibility of potentially contaminated toxin preparations prompted us to use toxin-converting phages (which to date have not been found to harbor two *slt* genes) or cloned toxin genes in *E. coli* K-12 strains for most purification purposes. Although *E. coli* strains that contain multiple copies of *slt*-I have not been identified (Table 3), the finding of multiple *slt*-II-related genes in EHEC strains argues strongly for the use of Southern analysis rather than colony hybridization to examine the distribution and types of toxins present in clinical isolates.

TABLE 5. Cytotoxicity of bacterial lysates for HeLa and Vero cells

Strain or plasmid[a]	Toxin	CD_{50}/ml for:		Vero/HeLa[b]
		Vero cells	HeLa cells	
E32511	SLT-II+IIc	10^5	10^4–10^5	1–10
pCKS109	SLT-II	10^4	10^4	1
pCKS111	SLT-IIc	10^3	10^2	10
pLP32	SLT-II	10^5	10^5	1
pJES101	SLT-IIv	10^7	10^2	10,000
S. dysenteriae	Shiga toxin	10^6	10^6	1

[a] The plasmids pCKS109 and pCKS111 contain toxin genes from E32511. All plasmids were maintained in nontoxinogenic *E. coli* DH5α. Assays were performed at least three times; data from a representative experiment are presented.
[b] Ratio of 50% cytotoxic dose per milliliter on Vero cells to that on HeLa cells.

Our detailed characterization of the two closely related toxin genes, *slt*-II and *slt*-IIc, from the *E. coli* O157:H$^-$ strain E32511 should clarify the literature concerning the nature of the toxin produced by E32511. The sequence homology between the two toxin operons in E32511 and *slt*-II was the basis for naming each operon *slt*-II, and the suffixes used to distinguish between genes for toxins that are very closely related but slightly different at the level of deduced amino acid sequence were consistent with Ito et al. (9) and Gannon et al. (4).

It is likely that the toxin genes encoding *slt*-II are present on a temperate bacteriophage. Willshaw et al. (48) reported the cloning of VT2 (E32511) from a 4.7-kb *Eco*RI fragment derived from bacteriophage ϕE32511. We found that the *slt*-II genes from E32511 were present on a 4.8-kb *Eco*RI fragment, whereas the *slt*-IIc genes were on a 5.5-kb *Eco*RI fragment. In addition, when we compared our restriction mapping data with those presented by Willshaw et al. (47) for ϕE32511, a *Hinc*II site was present in *slt*-II but absent from *slt*-IIc (Fig. 2, positions 1291 to 1296). The bacteriophages ϕE32511 and ϕ933W appear very similar morphologically, and both phages showed hybridization of *slt*-II-specific sequences to a 5-kb *Eco*RI fragment (33). It is possible that the *slt*-IIc genes are present on a bacteriophage that has not yet been isolated.

Contradictory results from neutralization assays with anti-SLT-II sera against crude extracts of strain E32511 have led to confusion and the apparently erroneous conclusion that SLT-II and VT2, which Scotland et al. defined as the toxin encoded by ϕE32511 (37a), are different toxins (5a). Dickie et al. and Head et al. reported only partial neutralization of E32511 toxin by anti-SLT-II (2, 5a). The presence of genes for two related but distinct toxins in E32511 could explain why Head et al. (5a) reported that antiserum directed against E32511 completely neutralized SLT-II toxin, even though anti-SLT-II only partially neutralized E32511 toxin. However, we and others have shown complete neutralization of E32511 toxin with antibodies directed against SLT-II (41, 49). These differences in neutralization patterns could be due either to variations in the strains used to generate anti-SLT-II (or anti-VT2) or to differential expression of the toxin genes under various growth conditions. Yutsudo et al. (49) showed that the VT purified from strain J-2 was immunologically identical to both the cytotoxin produced by an *E. coli*

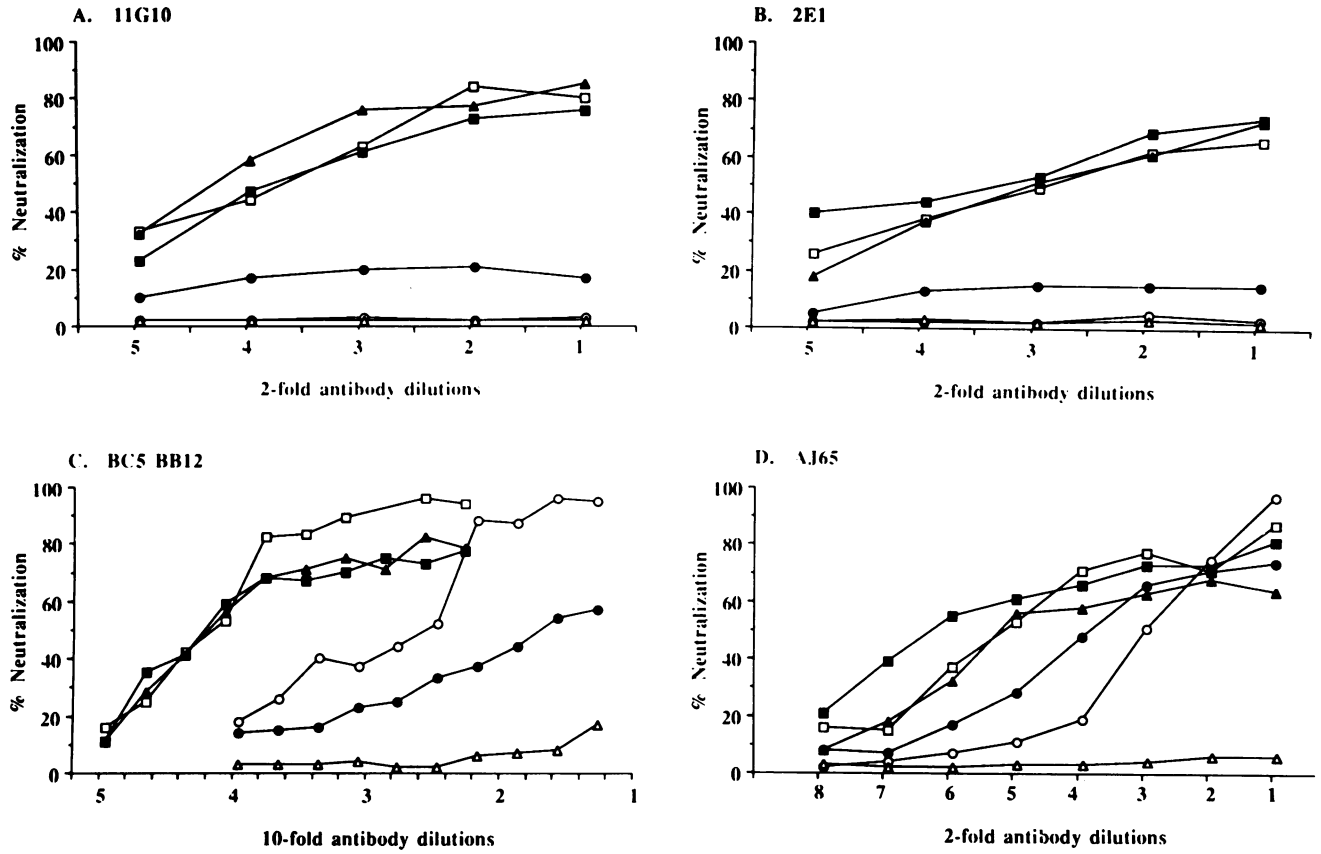

FIG. 4. Neutralization of toxins derived from E32511. Percent neutralization by several antibodies was determined by using bacterial lysates (see Materials and Methods for details). Antibodies used are as follows: SLT-II A subunit-specific antibodies 11G10 (A) and 2E1 (B), SLT-II B subunit-specific antibody BC5 BB12 (C), and SLT-II-specific polyclonal rabbit antiserum AJ65 (D). Assays were done at least three times; data from a representative experiment are presented. The plasmids pCKS109 and pCKS111 express, respectively, SLT-II and SLT-IIc cloned from E32511; the plasmids pLP32 and pJES101 express, respectively, SLT-II and SLT-IIv (35). All plasmids were maintained in nontoxinogenic *E. coli* DH5α. Symbols: □, E32511; ■, pCKS109; ●, pCKS111; ▲, pLP32; ○, pJES101; △, Shiga toxin.

K-12 strain lysogenized with φE32511 and SLT-II toxin, which supports our conclusion that the toxin genes present on φE32511 are *slt*-II and not *slt*-IIc.

Oku et al. (30) reported that purified toxin from B2F1 is 100-fold less cytotoxic for HeLa cells than for Vero cells. Although SLT-IIc is highly homologous to VT2vha of B2F1, we found that SLT-IIc was only slightly less cytotoxic for HeLa cells than for Vero cells. There are at least four possible explanations for this apparent discrepancy between our results and those of Oku et al. First, the toxin purified from B2F1 in 1989 (30) may have contained a mixture of VT2vha and VT2vhb, because at the time it was not recognized that B2F1 had two slightly different toxin operons. Second, the A subunit of SLT-IIc, which is very similar to but not identical to the A subunit of VT2vha, could be responsible for differences in relative toxicity of SLT-IIc and VT2vha for Vero and HeLa cells. Third, subtle changes between the conformations of VT2vha and SLT-IIc could alter the relative affinity or toxicity of the holotoxins for Vero and/or HeLa cells. Indeed, we found that monoclonal antibodies to either the A or B subunit of SLT-II could distinguish between SLT-II and SLT-IIc. Since the A subunits of SLT-IIc and SLT-II are identical but the B subunits vary, the B subunit amino acid differences between SLT-IIc and SLT-II must have altered the conformation of SLT-IIc sufficiently to affect reactivity with A subunit-specific monoclonal antibodies. A fourth explanation is that variations in the sensitivities of different HeLa cell lines to toxins may account for differences seen in relative cytotoxicities among laboratories.

Neutralization assays showed that the cytotoxic activity in sonically disrupted preparations of E32511 was phenotypically more like SLT-II than SLT-IIc. It is possible that *slt*-II could be expressed better than *slt*-IIc by E32511, although the only apparent difference in the regulatory regions of *slt*-II and *slt*-IIc was the first nucleotide of the −35 region. SLT-II could also be intrinsically more stable or have a higher specific activity than SLT-IIc. Alternatively, a pool of both types of B subunits, which is presumably present in E32511, may form heterologous pentameric B subunits that can be neutralized efficiently by antibodies directed against SLT-II.

The importance of multiple copies of *slt*-II in strains of *E. coli* associated with disease remains to be determined. However, it should be noted that Ostroff et al. (31) and Tarr et al. (43) found that *E. coli* O157:H7 strains that contained only *slt*-II genes were more frequently associated with HUS and thrombocytopenic purpura than strains containing *slt*-I or both *slt*-I and *slt*-II. In a study of *E. coli* O157 strains isolated in Great Britain, Scotland et al. (38) also noted that strains producing SLT-II alone were most frequently associated with HUS. A 3-year detailed study of HUS in children in the British Isles also showed a strong correlation between

SLT-II-producing strains and HUS (13, 23). Wadolkowski et al. found that SLT-II- but not SLT-I-producing *E. coli* strains were able to kill mice in the murine model system (45, 45a). Thus, a link has been established between the presence of *slt*-II genes and disease-causing *E. coli* O157 strains. Whether multiple copies of *slt*-II operons are responsible for enhanced virulence of an EHEC strain remains to be determined.

ACKNOWLEDGMENTS

These studies were supported in part by a grant from the National Institutes of Health (AI 20148-07) and a grant from the Uniformed Services University (RO73AK-02).

We thank Nancy A. Strockbine, Centers for Disease Control, Atlanta, Ga., for kindly providing the monoclonal antibody BC5 BB12 and Vernon L. Tesh for thoughtful discussions.

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