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Comparative Toxicity and Virulence of *Escherichia coli* Clones Expressing Variant and Chimeric Shiga-like Toxin Type II Operons

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Shiga-like toxin (SLT)-producing strains of *Escherichia coli* are known to cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans. The SLTs, particularly those related to type II (SLT-II), are a diverse family of toxins which may have differing in vitro or in vivo properties. To examine the impact of naturally occurring SLT-II sequence variation on the capacity of a given *E. coli* strain to cause disease, operons encoding four different SLT-II-related toxins, designated SLT-II/O111, SLT-II/OX3a, SLT-II/OX3b, and SLT-II/O48, were cloned in the same orientation in pBluescript. French pressure cell lysates of *E. coli* DH5α derivatives carrying these plasmids differed markedly in cytotoxicity for Vero cells, with 50% cytotoxic doses ranging from 20 to 328,000/ml. The strains also differed in oral virulence for streptomycin-treated mice, as judged by survival rate and/or median survival time, but virulence did not necessarily correlate with in vitro cytotoxicity. The SLT-II type associated with the lowest oral virulence was SLT-II/O111. Both the overall survival rate and the median survival time of mice challenged with clones producing this toxin were significantly greater than that for mice challenged with a clone producing the closely related SLT-II/OX3a. Experiments with clones carrying chimeric O111/OX3a SLT-II operons indicated that the reduced virulence was associated with an Arg-176→Gly substitution in the mature A subunit. Clones producing SLT-II/O48 and SLT-II/OX3b had similarly high cytotoxicities for Vero cells, but the latter was more virulent when fed to streptomycin-treated mice, as judged by median survival time. Experiments with clones carrying chimeric O48/OX3b SLT-II operons indicated that the increased virulence was a function of the A subunit of SLT-II/OX3b, which differs from the A subunit of SLT-II/O48 by only two amino acids (Met-4→Thr and Gly-102→Asp, respectively). These findings raise the possibility that naturally occurring SLT-II sequence variations may impact directly on the capacity of a given SLT-producing *E. coli* strain to cause disease.

Strains of *Escherichia coli* producing Shiga-like toxins (SLTs) are known to cause diarrhea and hemorrhagic colitis in humans. This can lead to potentially fatal systemic sequelae, such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (9, 14). Two major SLT types (SLT-I and SLT-II) have been described (14), and the genes encoding these were shown to be encoded by temperate bacteriophages (15). Diagnosis of SLT-producing *E. coli* (SLTEC) infection is complicated by the fact that such strains may be carried in the gut of apparently healthy individuals (19). However, there is an increasing body of evidence that SLTECs vary in their capacity to cause serious disease in humans and that this is a function of the type and/or amount of SLT produced. In one study, patients infected with SLTECs producing SLT-II alone were 6.8 times more likely to develop serious complications such as HUS or thrombotic thrombocytopenic purpura than those infected with SLTECs producing SLT-I alone or both SLT-I and SLT-II (16). Kleanthous et al. (10) also noted that 83% of SLTECs isolated in cases of HUS produced SLT-II only, but for SLTECs in cases of uncomplicated diarrhea or healthy controls, only 50% produced SLT-II only, the remainder producing both SLT-I and SLT-II. These findings are consistent with studies employing a streptomycin-treated mouse model of SLTEC infection (25). When mice were fed *E. coli* DH5α carrying an SLT-II operon cloned in a high-copy-number plasmid (pBluescript), their intestines became colonized by the recombinant strain and they subsequently died from acute renal cortical tubular necrosis. Mice fed *E. coli* DH5α carrying the parental plasmid or one containing an intact SLT-I operon were colonized but suffered no apparent ill effects. Similarly, mice fed a DH5α derivative carrying the SLT-II operon on a low-copy-number plasmid also survived (25). Oral administration of a clinical SLTEC isolate producing both SLT-I and SLT-II was fatal. However, mice could be protected by passive immunization with a monoclonal antibody to SLT-II but not with anti-SLT-I (25). These studies indicate that SLT-II is potentially more toxic in vivo than SLT-I.

In recent years, a large number of variations in the coding sequence for SLT-II have been described (3, 4, 6, 7, 20, 21, 23, 26), and these have generally been encoded chromosomally. Several chromosomally encoded variants of SLT-I have also been described (17, 18). It is possible that sequence variation may result in toxins with different properties, which could in turn impact on the capacity of the respective SLTEC to cause disease. Recently, Lindgren et al. (11) reported that an Asp→Asn substitution at position 16 in the mature B subunit was primarily responsible for large differences in the Vero cell cytotoxicity of four SLT-II-related toxins (SLT-II, SLT-IIc, SLT-IIvha, and SLT-IIvhb). However, they did not find differences in the intraperitoneal 50% lethal dose (LD_{50}) of purified toxins or in the oral virulence of *E. coli* clones expressing these toxins in the streptomycin-treated mouse model. The toxins

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studied by Lindgren et al. (11) differed from each other by at most four amino acids in the A subunit and two amino acids in the B subunit. We have been examining the sequence types of SLT-II genes from various SLTEC strains in our collection (20, 21), and some of these differ from classical SLT-II by as many as 16 and 13 amino acids in the A and B subunits, respectively.

In the present study, we have demonstrated significant differences in the relative cytotoxicity and oral virulence of otherwise isogenic *E. coli* clones expressing these SLT-II variants. Analysis of *E. coli* clones expressing chimeric toxin genes has shown that amino acid sequence variations in the A and B subunits are responsible for the observed effects on oral virulence and Vero cell cytotoxicity, respectively.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strains JM109 (28) and DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) were grown in Luria-Bertani (LB) broth (12) with or without 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). A spontaneous streptomycin-resistant derivative of DH5α (designated DH5αS) was isolated from an LB agar culture adjacent to a 100-μg streptomycin disc. Where appropriate, ampicillin and/or streptomycin was added to the growth medium at concentrations of 50 and 40 μg/ml, respectively.

SLT-II-related genes used in this study were derived from clinical SLTEC isolates. The OX3:H21 strain O31 was isolated in a case of sudden infant death syndrome and produces two SLT-II-related toxins, designated SLT-II/OX3a and SLT-II/OX3b. The O111:H$^-$ strain PH and the O48:H21 strain 94C were isolated in HUS cases, and each produced a single SLT-II-related toxin (designated SLT-II/O111 and SLT-II/O48, respectively; these strains also produced SLT-I-related toxins).

DNA manipulations. *E. coli* chromosomal DNA was extracted and purified as described previously (20). Plasmid DNA was extracted by the Triton X-100 lysis technique of Kahn et al. (8). Analysis of recombinant plasmids was carried out by digestion of DNA with one or more restriction enzymes under the conditions recommended by the supplier. Restricted DNA was electrophoresed in 0.8 to 1.5% agarose gels with a Tris-borate-EDTA buffer system as described by Maniatis et al. (12). Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (2).

PCR amplification and cloning of SLT-II operons. We have described previously the PCR amplification and cloning of the genes encoding SLT-II/OX3a, SLT-II/OX3b, and SLT-II/O111 (21). The same procedure was used in the present study to clone the gene encoding SLT-II/O48. Genomic DNA from the O48:H21 strain 94C was PCR amplified with two 17-mer oligonucleotide primers (5′-GATGGCGGTCCATTATC-3′ and 5′-AACTGACTGAATTGTGA-3′; designated P1 and P2, respectively). P1 is homologous to a region 177 to 194 bp upstream from the ATG initiation codon of the A subunit gene of SLT-II, while P2 is complementary to a region approximately 45 to 62 bp downstream from the SLT-II B subunit termination codon. Thus, P1 and P2 direct the amplification of an approximately 1.5-kb DNA fragment which incorporates the entire SLT-II operon, including the putative −10 and −35 promoter regions. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 94°C, 3 min of annealing at 47°C, and 4 min of elongation at 72°C. PCR products were band isolated after electrophoresis on 1.2% agarose gels, blunted by treatment with Klenow polymerase, cloned into the *Sma*I site of pBluescript SK (Stratagene, La Jolla, Calif.), and transformed into *E. coli* JM109. Reactivity of clones with SLT-II-specific probes was confirmed by Southern hybridization, and restriction analysis was used to identify those in which the SLT-II operon was inserted in the opposite orientation to the vector *lac* promoter. Recombinant plasmids expressing the genes encoding SLT-II/OX3a, SLT-II/OX3b, SLT-II/O111, and SLT-II/O48 were designated pJCP520, pJCP521, pJCP522, and pJCP523, respectively.

DNA sequence analysis. We have reported previously the nucleotide sequences of the genes encoding SLT-II/OX3a, SLT-II/OX3b, and SLT-II/O111 (20, 21). The sequence of the gene encoding SLT-II/O48 was determined by constructing nested deletion derivatives of pJCP523 by the method of Henikoff (5) with an Eraseabase kit (Promega Biotec, Madison, Wis.). These were transformed into *E. coli* JM109, and plasmid DNA was extracted from the resultant clones, as described above. Double-stranded DNA sequencing was then carried out with dye-labelled primers on an Applied Biosystems model 373A automated DNA sequencer. Sequence data were analyzed with DNASIS and PROSIS software version 7.0 (Hitachi Software Engineering, San Bruno, Calif.). For each of the SLT-II-related genes described above, the sequence was confirmed by analyzing a second copy cloned from an independent PCR, thereby confirming that the sequence of the cloned gene was identical to that of the wild-type gene.

Cytotoxicity assays. SLT cytotoxicity was assayed with Vero cells or HeLa cells, which were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and maintained in Dulbecco's modified Eagle's medium with 2% fetal calf serum. Confluent cell monolayers in 96-well flat-bottom microtiter trays were treated with filter-sterilized culture supernatant or French pressure cell lysates from the various clones, which had been serially diluted in maintenance medium. Cytotoxicity was assessed after 3 days of incubation at 37°C. The CD_{50} titer was defined as the maximum dilution of sample producing a cytotoxic effect on at least 50% of the cells of a given well.

Immunoblot quantitation of SLT-II-related proteins. Immunoblot analysis of serial dilutions of French pressure cell lysates was carried out with the SLT-II-specific monoclonal antibody 11E10 essentially as described by Lindgren et al. (11), except that a 4-chloro-1-naphthol–H_2O_2 substrate system was used.

In vitro translation inhibition assays. To determine the inhibitory effects of French pressure cell extracts on in vitro eukaryotic protein synthesis, 1 μl of each extract was added to reaction tubes containing 1.5 μl of tobacco mosaic virus RNA, 3.5 μl (3.5 μCi) of L-[4,5-^{3}H]leucine, and 20 μl of rabbit reticulocyte lysate (all obtained from Amersham, Buckinghamshire, England). After incubation for 60 min at 30°C, incorporation of label into trichloroacetic acid-insoluble material was determined as recommended by the supplier. Incorporation was expressed as a percentage of that obtained in the presence of *E. coli* DH5αS(pBluescript) lysate.

Mouse studies. The in vivo toxicity of French pressure cell extracts of clones producing various SLT-II-related toxins was estimated by intraperitoneal LD_{50}. Male BALB/c mice (6 to 8 weeks old) were injected intraperitoneally with 0.5-ml volumes of filter-sterilized extract serially diluted in phosphate-buffered saline. The LD_{50} was expressed as micrograms of lysate protein (measured by the method of Bradford [1]).

The oral virulence of *E. coli* clones expressing SLT-II-related toxins was determined with streptomycin-treated mice essentially as described by Wadolkowski et al. (25). Groups of up to eight male BALB/c mice (6 to 8 weeks old) were given drinking water containing 5 mg of streptomycin per ml for 24 h before challenge. Each mouse was then fed approximately 4×10^9 CFU of the appropriate *E. coli* DH5αS clone, suspended in 100 μl of 20% sucrose. After challenge, the drinking water was supplemented with 5 mg of streptomycin per ml and 5 mg of ampicillin per ml. Mice were monitored for 14 days, and the time of death was recorded. Kidneys were removed immediately postmortem or at the end of the experiment from representative mice from each group. These were fixed in 10% buffered formalin and examined histologically after routine processing and staining with hematoxylin and eosin. Intestinal colonization of mice with the respective *E. coli* clone was also monitored by culturing either feces or bowel contents on LB agar supplemented with ampicillin and streptomycin.

Statistical analysis. Differences in survival times and overall survival rates between groups of mice fed different *E. coli* clones were analyzed by the Mann-Whitney U test and the Fisher exact probability test, respectively.

Nucleotide sequence accession numbers. The nucleotide sequence of the gene encoding SLT-II/OX3a has been deposited with the EMBL database (accession number X65949), while those for the genes encoding SLT-II/OX3b, SLT-II/O111, and SLT-II/O48 have been deposited with GenBank (accession numbers L11079, L11078, and Z37725, respectively).

RESULTS

Construction of *E. coli* clones expressing SLT-II-related genes. The purpose of this study was to compare the biological properties of otherwise identical *E. coli* clones expressing different SLT-II-related genes. To achieve this, four different SLT-II-related operons were PCR amplified, and 1.5-kb amplicons were blunted and cloned into the *Sma*I site of pBluescript SK as described in Materials and Methods. The DNA inserts in these plasmids were sequenced either previously or as part of this study. The deduced amino acid sequences for the various SLT-II-related genes, aligned with the published sequence for SLT-II encoded by phage 933W (7), are shown in Fig. 1. SLT-II/OX3b and SLT-II/O48 were more closely related to classical SLT-II than were SLT-II/OX3a and SLT-II/O111. Of the published SLT-II variant sequence types, the former two toxins are most closely related to SLT-IIc (23). SLT-II/OX3a was significantly different from SLT-II, sharing only 95.6% identity in the A subunit and 85.9% in the B subunit.

Cytotoxicity and virulence of SLT-II-producing *E. coli* clones. In view of the report by Lindgren et al. (11) that SLT-II-related toxins which differed in Vero cell cytotoxicity had similar mouse lethalities, a pilot experiment was conducted to determine whether there were differences in the in vivo or in vitro properties of the SLT-II variants in the present study. French pressure cell lysates of *E. coli* JM109 harboring the various constructs were tested for Vero cell cytotoxicity

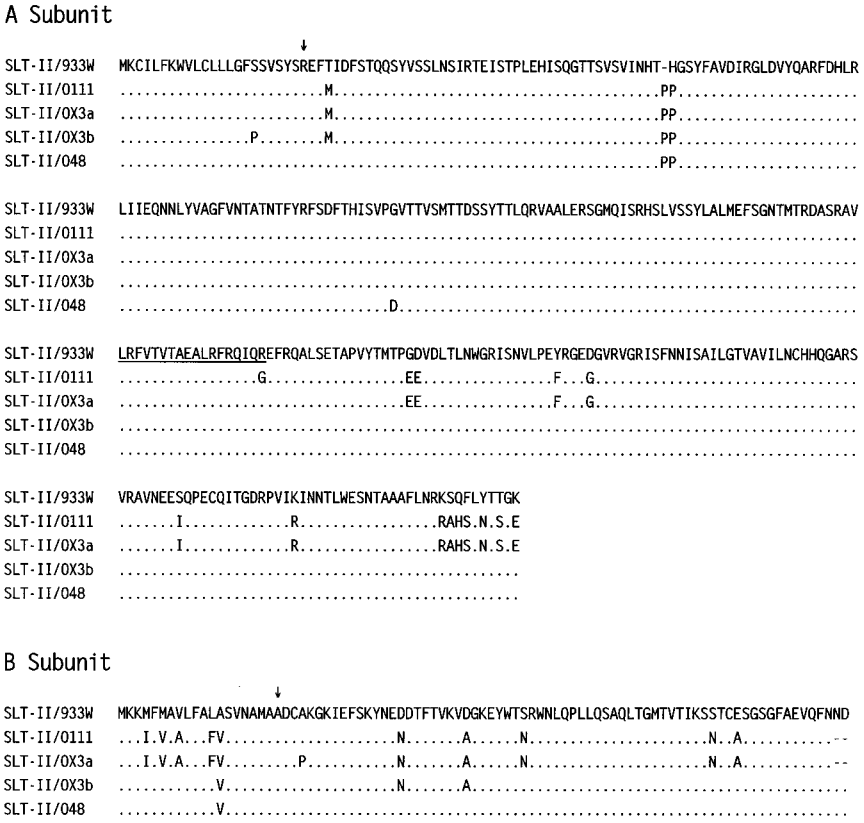

FIG. 1. Deduced amino acid sequences of A and B subunits of the various SLT-II-related toxins are aligned with the published sequence for SLT-II from bacteriophage 933W (7). The 18-amino-acid region which is conserved between SLT-I and SLT-II is underlined. Dots denote residues identical to SLT-II/933W; dashes denote absent residues. Arrows indicate the first residue of the mature polypeptide for each subunit.

and mouse intraperitoneal lethality (Table 1). There was a wide variation in the toxicity of the various crude extracts, with CD_{50} titers ranging from 40 to 81,920 and LD_{50}s ranging from 100 to 1,600 μg. Although not related linearly, Vero cytotoxicity roughly paralleled intraperitoneal toxicity. These differences were not due to variations in growth of the *E. coli* clones or efficiency of lysis or to plasmid copy number, since total protein concentrations in the filtered lysates and plasmid yields were not significantly different (data not shown). The variations were also not due to differences in the proportion of toxin released from the cells, since the Vero cell CD_{50} titers of culture supernatants paralleled those of the respective cell lysate, except that the titers were two- to fourfold lower (data not shown). Thus, for each SLT-II variant, the majority of the toxin remained cell associated.

To further examine the apparent differences in toxicity, the various plasmids were transformed into *E. coli* DH5αS. Approximately 4×10^9 CFU of the various clones were then fed to groups of three to four streptomycin-treated mice (Table 1). All mice receiving the clones expressing either SLT-II/OX3a, SLT-II/O48, or SLT-II/OX3b died, but those receiving the DH5αS derivative expressing SLT-II/O111 or DH5αS carrying pBluescript suffered no apparent ill effects. In spite of the small group sizes in this pilot experiment, the difference in survival rates between mice challenged with DH5αS expressing SLT-II/O111 and those challenged with clones expressing other

TABLE 1. In vitro and in vivo toxicities of *E. coli* clones expressing SLT-II variants

Plasmid	Toxin	Toxicity of *E. coli* lysate[a]		Oral challenge[b]	
		Vero cell CD_{50} (titer/ml)	i.p. LD_{50} (μg)	No. of deaths/ no. challenged	Median survival time (days)
pBluescript	Nil	ND[c]	Not lethal	0/3	>14
pJCP522	SLT-II/O111	40	1,600	0/4	>14
pJCP520	SLT-II/OX3a	320	300	4/4	6.5
pJCP523	SLT-II/O48	41,000	200	3/3	7.5
pJCP521	SLT-II/OX3b	82,000	100	3/3	4.7

[a] Vero cell CD_{50} (titer per milliliter) and intraperitoneal (i.p.) LD_{50} (expressed as micrograms of protein) of French pressure cell lysates of *E. coli* JM109 carrying the indicated plasmids.
[b] Mice were fed *E. coli* DH5αS derivatives carrying the indicated plasmids.
[c] ND, no detectable cytotoxicity ($CD_{50} < 10$).

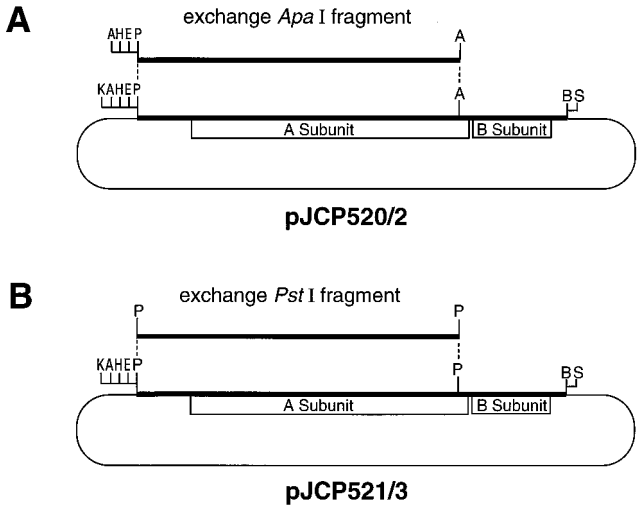

FIG. 2. Scheme for construction of chimeric SLT-II operons. (A) Construction of chimeric OX3a/O111 operons by exchanging *Apa*I fragments between pJCP520 and pJCP522; (B) construction of chimeric OX3b/O48 operons by exchanging PstI fragments between pJCP521 and pJCP523. The solid lines represent the cloned PCR product containing the SLT-II-related operon, with boxes indicating the regions encoding the A and B subunits. The thin lines represent pBluescript DNA. Restriction site abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I.

SLT-II variants was significant ($P < 0.05$). Also, all three mice challenged with DH5αS expressing SLT-II/OX3b died before any of the mice challenged with clones expressing the other toxins ($P \leq 0.05$). Thus, the pilot experiment suggested that naturally occurring sequence variations in SLT-II-related toxins could affect either overall survival rate or survival time in the streptomycin-treated mouse model.

Cytotoxicity and virulence of *E. coli* DH5αS expressing native and chimeric SLT-II operons. The results described above indicated that *E. coli* DH5αS producing SLT-II/O111 was significantly less virulent than that expressing other SLT-II operons, including SLT-II/OX3a, which differed by only one amino acid in the A subunit and one amino acid in the B subunit (Fig. 1). Also, clones expressing SLT-II/OX3b were significantly more virulent than those expressing other SLT-II operons, including SLT-II/O48, which differed by only two amino acids in each of the A and B subunits. To determine which amino acid variations were impacting on virulence, four chimeric SLT-II operons were constructed. The genes for SLT-II/OX3a and SLT-II/O111 both contain a single *Apa*I site 30 bp upstream of the A subunit termination codon. The single A subunit amino acid difference between the two toxins is upstream of this site. Therefore, chimeric OX3a/O111 operons were constructed by digesting pJCP520 and pJCP522 with *Apa*I and band purifying the resultant 1.17-kb fragments (containing the promoter and the A subunit coding region) and the 3.32-kb fragments (containing the vector and the B subunit coding region), as shown in Fig. 2A. Heterologous large and small fragments were then mixed, ligated, and transformed into *E. coli* DH5αS. Transformants containing heterologous A subunit inserts were identified by hybridization with a digoxigenin-labelled A subunit-specific probe, and plasmid DNA was extracted from these clones and subjected to restriction analysis to confirm correct orientation of the insert. Representative plasmids encoding chimeric SLT-II/O111 A subunit plus SLT-II/OX3a B subunit and SLT-II/OX3a A subunit plus SLT-II/O111 B subunit operons were designated pJCP527 and pJCP528, respectively. Chimeric OX3b/O48 SLT-II operons were constructed in an analogous manner, exploiting a unique *Pst*I site in the inserts of pJCP521 and pJCP523 51 bp upstream of the A subunit termination codon (Fig. 2B). Again, the A subunit amino acid differences between SLT-II/OX3b and SLT-II/O48 are located upstream of the codon containing this restriction site (Fig. 1). Plasmids encoding chimeric SLT-II/O48 A subunit plus SLT-II/OX3b B subunit and SLT-II/OX3b A subunit plus SLT-II/O48 B subunit operons were designated pJCP529 and pJCP530, respectively.

TABLE 2. Cytotoxicities of *E. coli* DH5αS expressing native and chimeric SLT-II operons[a]

Plasmid	A subunit	B subunit	CD_{50} (titer/ml)	
			Vero cell	HeLa cell
pBluescript	Nil	Nil	ND[b]	ND
pJCP522	O111	O111	20	ND
pJCP520	OX3a	OX3a	160	ND
pJCP523	O48	O48	328,000	160
pJCP521	OX3b	OX3b	164,000	40
pJCP527	O111	OX3a	20	ND
pJCP528	OX3a	O111	160	ND
pJCP529	O48	OX3b	82,000	10
pJCP530	OX3b	O48	164,000	80

[a] Vero cell and HeLa cell CD_{50}s were determined for French pressure cell lysates of *E. coli* DH5αS carrying the indicated plasmids.
[b] ND, no detectable cytotoxicity ($CD_{50} < 10$).

The Vero and HeLa cell cytotoxicities of French pressure cell lysates of *E. coli* DH5αS expressing the various native and chimeric SLT-II operons are shown in Table 2. These results confirmed the earlier findings that clones expressing SLT-II/O111 were eightfold less cytotoxic for Vero cells than those expressing SLT-II/OX3a. They also demonstrated that the amino acid substitution in the A subunit, rather than the B subunit, was responsible for this reduced toxicity, since the cytotoxicity of the *E. coli* DH5αS(pJCP527) lysate was eightfold lower than that of the DH5αS(pJCP528) lysate. The capacity of the various lysates to inhibit eukaryotic protein synthesis in vitro was also determined as described in Materials and Methods. The DH5αS(pJCP522) and DH5αS(pJCP527) lysates inhibited [^{3}H]leucine incorporation by 26 and 6%, respectively, while the DH5αS(pJCP520) and DH5αS(pJCP528) lysates inhibited [^{3}H]leucine incorporation by 82 and 71%, respectively. These results imply that the reduced toxicity associated with the single amino acid substitution in the A subunit of SLT-II/O111 is due to a direct effect on the capacity of the toxin to inhibit protein synthesis (i.e., catalytic activity).

There were only slight differences in the Vero cell CD_{50} titers of lysates of DH5αS expressing SLT-II/OX3b, SLT-II/O48, or either of the two chimeric derivatives of these toxins (encoded by pJCP529 and pJCP530). Although the HeLa cell CD_{50} titers were considerably lower than the respective Vero cell titers, a more pronounced difference in cytotoxicity was observed. Lysates of DH5αS(pJCP523) (expressing SLT-II/O48) were fourfold more toxic to HeLa cells than DH5αS (pJCP521) (expressing SLT-II/OX3b). This increased toxicity was associated with the B subunit of SLT-II/O48, since DH5αS (pJCP530) lysates were eightfold more cytotoxic to HeLa cells than those of DH5αS(pJCP529) (Table 2). To eliminate the possibility that the differences in cytotoxicity described above were due to variable levels of expression of the SLT-II-related operons, immunoblot analysis was carried out on serial twofold dilutions of lysates from each clone with an SLT-II-specific monoclonal antibody. No obvious differences in the intensity of

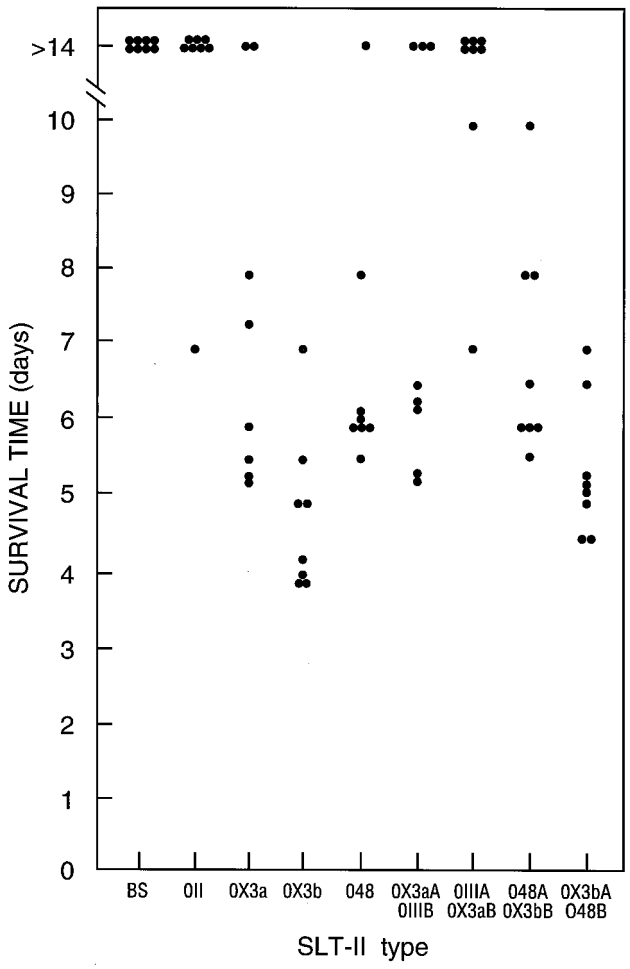

FIG. 3. Survival times of streptomycin-treated mice after oral infection with *E. coli* DH5αS derivatives expressing native and chimeric SLT-II operons. Mice were fed approximately 4×10^9 CFU of DH5αS carrying pBluescript (BS), pJCP522 (O111), pJCP520 (OX3a), pJCP521 (OX3b), pJCP523 (O48), pJCP528 (OX3aA + O111B), pJCP527 (O111A + OX3aB), pJCP529 (O48A + OX3bB), or pJCP530 (OX3bA + O48B) and monitored for 14 days.

labelling were observed for any of the clone lysates (data not shown).

Groups of eight mice were then challenged orally with approximately 4×10^9 *E. coli* DH5αS organisms carrying the various recombinant plasmids, and the survival time of each mouse was recorded (Fig. 3). Fecal samples were collected from two mice, selected at random from each group, on days 1 to 4 after challenge, for quantitation of SLT-II-producing *E. coli* DH5αS derivatives. All DH5αS clones colonized the gut with similar efficiencies, with levels for all clones within the range of 5.0×10^8 to 3.2×10^9 CFU/g of feces after 1 day. Numbers were maintained within this range throughout the 4-day period (data not shown).

The mouse virulence of the clones producing the native toxins was consistent with that observed in the pilot experiment described in Table 1. DH5αS(pJCP522) (expressing SLT-II/O111) was the least-virulent strain. Seven of the eight mice survived, compared with two of eight, none of eight, and one of eight survivors for mice challenged with clones expressing SLT-II/OX3a, SLT-II/OX3b, and SLT-II/O48, respectively (P values of <0.025, <0.005, and <0.01, respectively). Six of the eight mice challenged with DH5αS(pJCP527) (expressing the chimeric O111 A subunit plus OX3a B subunit toxin) survived, and this was not significantly different from the survival rate for those challenged with DH5αS(pJCP522). However, the median survival time for mice challenged with DH5αS(pJCP527) (>14 days) was significantly greater than that for mice challenged with DH5αS(pJCP520) expressing SLT-II/OX3a (6.6 days; $P < 0.02$) or DH5αS(pJCP528) expressing the chimeric OX3a A subunit plus O111 B subunit toxin (6.3 days; $P < 0.05$). In addition, the median survival time of mice challenged with DH5αS(pJCP528) was significantly lower than that of mice challenged with the clone producing SLT-II/O111 (>14 days; $P < 0.03$). Thus, the differences in both the Vero cell cytotoxicity and the oral virulence between SLT-II/O111 and SLT-II/OX3a are due to the single amino acid difference in the A subunit rather than the single amino acid difference in the B subunit.

Of the clones producing native SLT-II-related toxins, DH5αS(pJCP521) (expressing SLT-II/OX3b) was the most virulent. The median survival time of mice challenged with this strain was 4.5 days, which was significantly less than that of mice challenged with the clones expressing SLT-II/OX3a (6.6 days; $P < 0.0025$), SLT-II/O48 (5.9 days; $P < 0.002$), and SLT-II/O111 (>14 days; $P < 0.001$). The significant difference in median survival times of mice challenged with clones producing SLT-II/OX3b rather than SLT-II/O48 is of particular interest because the two strains both have high cytotoxicities for Vero cells (CD_{50}s, 164,000 and 328,000/ml, respectively) and the toxins differ by only two amino acids in each of the A and B subunits. It is also interesting that the HeLa cell cytotoxicity was fourfold higher for the less-virulent DH5αS (pJCP523) (Table 2) than for DH5αS(pJCP521). The increased virulence of SLT-II/OX3b was associated with the A subunit, as there was no significant difference between the median survival times of mice challenged with DH5αS (pJCP530) (producing the chimeric OX3b A subunit plus O48 B subunit toxin) and those challenged with DH5αS(pJCP521) (median survival times, 5.0 and 4.5 days, respectively). Moreover, the median survival time of mice challenged with the former strain was significantly less than that for mice challenged with DH5αS(pJCP523) (5.9 days; $P < 0.02$) or DH5αS(pJCP529) (producing the chimeric O48 A subunit + OX3b B subunit toxin [6.2 days; $P < 0.01$]).

Histological studies. Histological examination of kidneys from mice succumbing to challenge with the various SLT-II-producing strains indicated signs of renal tubular damage, whereas no abnormalities were detected in kidneys from mice challenged with DH5αS(pBluescript) (Fig. 4A). The glomeruli appeared normal in routine paraffin sections. Tubular damage included extensive epithelial cell vacuolation, tubular necrosis with nuclear debris, and proteinaceous exudate in the lumen (Fig. 4B and C) as well as gross flattening of the tubular epithelial cells with concomitant dilation of the lumen. No obvious correlation was seen between the degree of damage and the toxin type produced by the challenge strain. Interestingly, clear signs of renal damage, including, in some cases, gross flattening of tubular epithelial cells and dilation of the lumen, were seen in kidney sections from mice which survived challenge with clones producing the less-virulent SLT-II types (Fig. 4D). These mice were alive and apparently well when the experiment was terminated 14 days after challenge. Clearly, gross tubular necrosis is not necessarily incompatible with survival. The regenerative capacity of renal tubular function is well known, and Tesh et al. (24) reported histological evidence of such regeneration in the kidneys of a mouse that had been injected with SLT-II 3 days earlier (although this particular mouse was reported to be ill at the time of sacrifice).

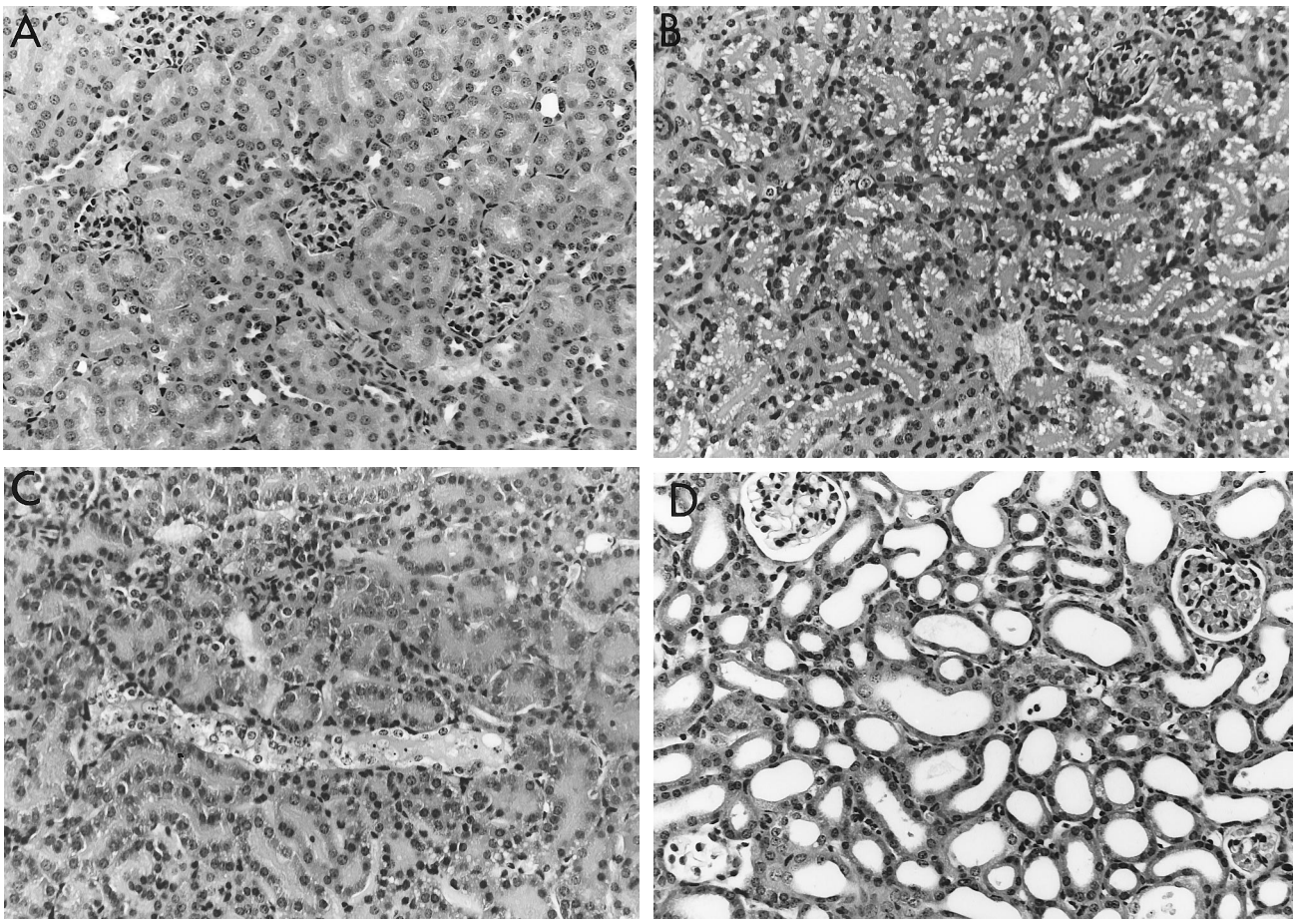

FIG. 4. Photomicrographs of the renal cortex from a healthy control mouse 14 days after oral challenge with DH5αS(pBluescript), showing normal glomeruli and tubules (A); a mouse which died 4.8 days after challenge with DH5αS(pJCP530), showing marked vacuolation of tubular epithelial cells (B); a mouse which died 5.9 days after challenge with DH5αS(pJCP523), showing tubular necrosis with nuclear debris and proteinaceous exudate in the tubule lumen (C); and a mouse which was alive and apparently well 14 days after challenge with DH5αS(pJCP528), but showing gross flattening of tubular epithelial cells and dilation of the tubule lumen (D). Sections were stained with hematoxylin and eosin. Magnifications, ×160 (A, B, and C) and ×257 (D).

Construction and cytotoxicity of OX3a/O48 SLT-II chimeras. A noteworthy feature of the study described above was that while there was a clear difference in the levels of virulence (as judged by median survival time) between strains producing SLT-II/O48 and SLT-II/OX3b in spite of similar Vero cell cytotoxicities, the former strain was no more virulent than the DH5αS derivative producing SLT-II/OX3a, which was about 10^3-fold less cytotoxic for Vero cells (Table 1 and Fig. 3). These two toxins differ by 15 amino acids in the A subunit and 8 amino acids in the mature B subunit. Chimeric toxin genes were constructed to determine which subunit was responsible for the observed difference in in vitro toxicity. Sequence variation at the 3′ end of the A subunit genes of the two toxins precluded the use of the *Pst*I or *Apa*I sites for the construction of the chimeras. To overcome this, PCR was used to amplify separately the promoter plus A subunit and the B subunit coding regions from pJCP520 and pJCP523 and at the same time to introduce restriction sites to permit reassembly of heterologous fragments into intact chimeric SLT-II operons, as shown in Fig. 5. The resultant 1,184- and 353-bp amplicons were band purified after agarose gel electrophoresis. The DNA fragments were then digested with *Nde*I, and heterologous A and B subunit fragments were combined and ligated. The ligation mix was then digested with *Eco*RI and ligated with *Eco*RI-digested pBluescript SK. This mix was used to transform *E. coli* JM109, and transformants containing heterologous A and B subunit inserts were identified by hybridization with digoxigenin-labelled A subunit-specific and B subunit-specific oligonucleotide probes. Plasmid DNA was extracted from these clones and subjected to restriction analysis using *Eco*RI, *Nde*I, *Eco*RI-*Nde*I, and either *Pst*I or *Apa*I to confirm that a complete SLT-II operon had been reconstituted and to determine the orientation of the 1.5-kb *Eco*RI insert with respect to the vector promoter (data not shown). Representative plasmids encoding chimeric O48 A subunit plus OX3a B subunit SLT-II operons and OX3a A subunit plus O48 B subunit SLT-II operons, inserted in the opposite orientation to the pBluescript *lac* promoter, were designated pJCP531 and pJCP532, respectively. The nucleotide substitutions required for creation of the *Nde*I site did not affect the deduced amino acid sequence of the B subunits, the B subunit ribosome binding site, or the predicted RNA secondary structure for the chimeric operons.

The Vero cell cytotoxicities of French pressure cell lysates of *E. coli* JM109 carrying the plasmids described above or the plasmids encoding SLT-II/OX3a or SLT-II/O48 are shown in Table 3. These results indicate that it is the B subunit of SLT-II/O48 which is responsible for the increased Vero cell

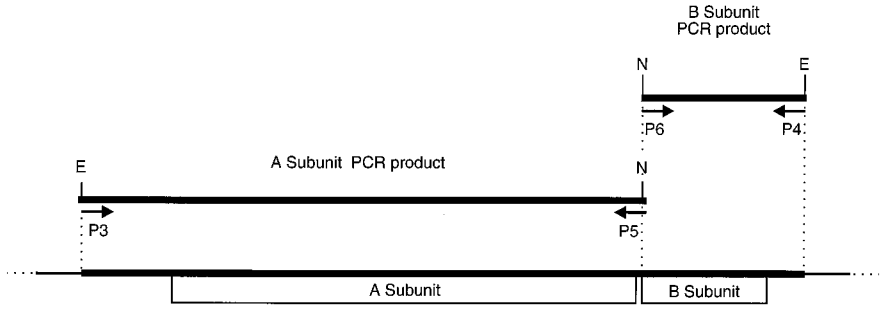

FIG. 5. Scheme for PCR amplification of pJCP520 and pJCP523 DNA yielding 1,184-bp promoter plus A subunit coding regions and 353-bp B subunit coding regions, by use of primer pairs P3/P5 and P4/P6, respectively. P3 is the same as P1, but with the addition of 5′-GCCGGAATTC (which contains an *Eco*RI site [underlined]) to the 5′ end. Primer P5 (5′-ATCTTCTTCATATGTAACTCCT) anneals to the region of the plus strand of either pJCP520 or pJCP523 spanning the B subunit initiation codon and introduces a *Nde*I site (underlined) at this point. P4 is the same as P2, but with the addition of the *Eco*RI linker 5′-GCCGGAATTC to the 5′ end. P6 (5′-AGGAGTTACATATGAAGAAGAT) anneals to the B subunit initiation codon region of the minus strand and also introduces a *Nde*I site (underlined) at this point. The location of *Nde*I (N) and *Eco*RI (E) sites incorporated during the PCR are indicated on the diagram.

cytotoxicity. The amino acid differences between the mature B subunits of SLT-II/OX3a and SLT-II/O48 include Asn rather than Asp, respectively, at position 16. This substitution has been shown previously to reduce the Vero cell cytotoxicity of SLT-II-related toxins approximately 100-fold (11).

DISCUSSION

In the present study, we have examined the oral virulence of *E. coli* DH5αS derivatives expressing various SLT-II-related genes, cloned in the same orientation in pBluescript. The results clearly demonstrate that virulence in the streptomycin-treated mouse model does not necessarily correlate with in vitro cytotoxicity. In one case, strains differing in Vero cell cytotoxicity by a factor of 500 had indistinguishable oral virulence. On the other hand, significant differences in oral virulence were observed between strains with the same in vitro cytotoxicities.

The SLT-II type associated with the lowest oral virulence was SLT-II/O111. Both the overall survival rate and median survival time of mice challenged with clones producing this toxin were significantly greater than that of mice challenged with a clone producing the closely related SLT-II/OX3a. The increased survival of mice in a given group was not a result of sporadic failure of the challenge strain to colonize the gut, since high levels of the respective DH5αS derivative (approximately 10^9 CFU/g) were isolated from the gut contents of each mouse, either at the time of death or at the termination of the experiment (data not shown). SLT-II/O111 and SLT-II/OX3a differ by only two amino acids (Gly instead of Arg at residue 176 in the mature A subunit and Ala instead of Pro at residue 4 in the mature B subunit). Experiments with the chimeric SLT-II-producing DH5αS derivatives indicated that both the reduced Vero cell cytotoxicity and the reduced oral virulence of clones producing SLT-II/O111 were associated with the A subunit, i.e., with Gly-176. Interestingly, A subunit residue 176 is located at the C-terminal end of an 18-amino-acid sequence, which is identical for SLT-I, SLT-II, and all previously reported variants of SLT-II. This sequence spans one of the three regions of the SLT A subunit which have been shown to have homology with ricin (27). All three regions in the ricin A subunit have been mapped to a cleft which forms the putative active site, as determined by X-ray crystallographic analysis (13). Therefore, any amino acid change within or flanking any of these regions has the potential to modify the catalytic activity or substrate binding efficiency of these toxins. Yamasaki et al. (27) examined the effects of site-directed mutagenesis of a number of amino acids in the analogous regions of SLT-I, and mutation of several of these (particularly Glu-167, Arg-170, and Arg-172) reduced cytotoxicity significantly. However, the effect of mutation of Arg-176 was not investigated. The lower in vitro and in vivo toxicity of recombinant *E. coli* expressing SLT-II/O111 with respect to otherwise identical constructs expressing SLT-II/OX3a is strongly suggestive that the residue at position 176 of SLT-II is important for catalytic function. This conclusion is supported by the finding that the presence of Gly instead of Arg at this position correlated with a significantly reduced capacity of recombinant *E. coli* lysates to inhibit in vitro eukaryotic protein synthesis.

TABLE 3. Cytotoxicities of *E. coli* JM109 expressing native and chimeric SLT-II operons[a]

Plasmid	A subunit	B subunit	Vero cell CD_{50} (titer/ml)
pJCP520	OX3a	OX3a	160
pJCP523	O48	O48	82,000
pJCP531	O48	OX3a	40
pJCP532	OX3a	O48	164,000

[a] Vero cell CD_{50} titers were determined for French pressure cell lysates of *E. coli* JM109 carrying the indicated plasmids.

The two clones with the greatest cytotoxicity for Vero cells were those producing SLT-II/OX3b and SLT-II/O48, and these are more closely related to classical SLT-II than SLT-II/OX3a or SLT-II/O111. The mature A subunits of SLT-II/OX3b and SLT-II/O48 both differ from SLT-II by three residues. The mature B subunit of SLT-II/O48 is identical to SLT-II, while that of SLT-II/OX3b differs by two residues. The SLT-II/OX3b B subunit is identical to that published for SLT-IIc (23). As mentioned previously, Lindgren et al. (11) have reported that Asp-16 of the SLT-II B subunit is responsible for a 100-fold-greater cytotoxicity over that of SLT-IIc, which has Asn at this position. In the present study, we did not observe a significant difference in the Vero cell CD_{50} of lysates of clones expressing SLT-II/OX3b or SLT-II/O48. However, the relative cytotoxicities of the lysates for HeLa cells suggested that SLT-II/O48 was slightly more toxic for this cell line, and the experiments with O48/OX3b SLT-II chimeras indicated that the B subunit was responsible for this, as would be expected from the findings of Lindgren et al. (11).

Remarkably, however, DH5αS producing SLT-II/OX3b was significantly more virulent in the streptomycin-treated mouse

model than DH5αS producing SLT-II/O48, as judged by median survival times, even though both strains colonized the gut at similar levels (approximately 10^9 CFU/g of feces). Moreover, the experiments with clones expressing O48/OX3b chimeric toxins demonstrated that the significantly increased virulence was a function of the A subunit. The DNA fragments exchanged during construction of the chimeric operons of course included the noncoding region 5′ to the A subunit initiation codon, which includes the A subunit gene ribosome binding site as well as −10 and −35 promoter regions. However, there are no differences in nucleotide sequence between pJCP521 and pJCP523 in these regions (data not shown), and this eliminates the possibility of different in vivo levels of transcription for the two operons. There are only two amino acid differences between the mature A subunits of SLT-II/OX3b and SLT-II/O48, i.e., Met versus Thr, respectively, at position 4, and Gly versus Asp, respectively, at position 102. Neither of these residues has been associated previously with effects on cytotoxicity, although Perera et al. (22) reported that deletion of residues 3 to 18 inclusive abolished the catalytic activity of the SLT-II A subunit.

The reason for the increased oral virulence of clones producing SLT-II/OX3b rather than SLT-II/O48 is not known. Effects on catalytic activity of the A subunit are unlikely to be responsible, since this would be expected to be reflected in Vero cell cytotoxicity (which was not observed). Moreover, lysates of both of these clones inhibited in vitro protein synthesis to similar extents (approximately 70% inhibition of [^{3}H]leucine incorporation). Differences in absorption of toxin by the gut mucosa or in efficiency of toxin internalization are possible alternative explanations. Sequence differences in the A subunit might also impact on in vivo holotoxin stability, which is not manifested in vitro.

Differences in median survival times after oral infection with *E. coli* strains producing different SLT-II-related toxins may be of considerable significance during natural SLTEC infection. In the present study, antibiotic selection maintained high numbers of SLTEC in the gut (approx. 10^9 CFU/g) for the duration of the experiments. This is not the case in natural infections, where SLTEC numbers may be high during the acute phase but diminish rapidly thereafter, presumably as a consequence of a local immune response to the SLTEC. Our own experience with HUS cases is that during the acute phase, the causative SLTEC may represent as much as 95% of the organisms growing on MacConkey agar fecal cultures, but specimens collected 1 week later are often negative for SLTECs, when tested with a sensitive PCR assay capable of detecting 10 CFU of SLTEC against a background of 10^9 other organisms (19). Thus, SLTECs producing more-potent (faster-acting) SLT-II-related toxins may be much more likely to induce serious renal injury during the transient period of high colonization, resulting in progression from diarrhea to HUS. The pathogenesis of SLTEC infection in humans is undoubtedly multifactorial, involving a combination of toxin and accessory virulence factors such as adhesins, hemolysins, etc. Nevertheless, the findings of the present study indicate that when considered in isolation, naturally occurring SLT-II sequence variations may have a direct impact on the capacity of a given SLTEC to cause disease.

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