Virulence of Enterohemorrhagic *Escherichia coli* O91:H21 Clinical Isolates in an Orally Infected Mouse Model

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Escherichia coli K-12 strains producing high levels of Shiga-like toxin type II (SLT-II) but not SLT-I were previously shown to be virulent in an orally infected, streptomycin-treated mouse model. In this investigation, we tested the virulence of several SLT-II-producing enterohemorrhagic *E. coli* (EHEC) isolates from patients with hemorrhagic colitis or hemolytic uremic syndrome. All of the strains tested were able to colonize the mouse intestine. However, only two strains were consistently virulent for mice: O91:H21 strain B2F1(Str^r), which was previously shown to carry two copies of *stx*-II-related toxins, and O91:H21 strain H414-36/89(Str^r), which was found in this study to contain three genes from the *stx*-II group. The oral 50% lethal doses of strains B2F1(Str^r) and H414-36/89(Str^r) when fed to streptomycin-treated mice were less than 10 bacteria. Histological sections from moribund mice fed the O91:H21 strains demonstrated extensive renal tubular necrosis; however, hematological results were not consistent with a diagnosis of hemolytic uremic syndrome. The central role of SLT in the virulence of the O91:H21 EHEC strains was supported by the finding that streptomycin-treated mice preinoculated with monoclonal antibody specific for SLT-II survived oral challenge with either B2F1(Str^r) or H414-36/89(Str^r). The basis for the variation in virulence among the SLT-II-producing EHEC strains tested was not determined. However, a correlation between the capacity of an EHEC strain to grow in small intestinal mucus and lethality in the streptomycin-treated mice was observed.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a family of pathogenic *E. coli* that colonize the large bowel and have been isolated from patients with diarrhea, hemorrhagic colitis (33, 48, 49), and/or hemolytic uremic syndrome (HUS) (23, 29, 33, 46). Serotypes of prototypic EHEC strains include O157:H7, O157:H⁻, and O26:H11 (25, 26). Three features are characteristic of these prototypic strains. First, they harbor plasmids of approximately 60 MDa that are similar but not necessarily identical among all EHEC strains (2). The role(s) of this large plasmid(s) in virulence and/or adherence remains to be determined; reported data describing a role for the plasmid in adherence are conflicting (19, 20, 60, 61). Second, prototypic EHEC strains contain a chromosomally located *eae* locus (18, 67) that has been associated with intimate bacterial attachment to host cells by another group of diarrheagenic *E. coli*, enteropathogenic *E. coli*, and may also be important in EHEC adherence (for reviews, see references 5 and 58). Third, EHEC strains, whether or not they are of the prototypic serotype, all produce Shiga-like toxins (SLTs), also known as verotoxins (VT) (for reviews, see references 11, 22, 39, and 57).

SLTs are bipartite molecules that consist of an enzymatically active A subunit and five binding (B) subunits (39). SLTs inhibit protein synthesis and are cytotoxic for both HeLa and Vero cells (7, 10, 42). In some clinical isolates, the SLT genes are located on toxin-converting lysogenic phage (40, 41, 52, 54, 65). Two antigenically distinct groups of SLTs have been described, SLT-I and SLT-II, which share 64% deduced amino acid sequence homology (17). Within the last few years, several new SLTs have been identified which are considered members of the SLT-II group on the basis of sequence homology and partial serological cross-reactivity with SLT-II. Among these new SLT-II group

members are SLT-IIva (9), SLT-IIvha (VT2ha), SLT-IIvhh (VT2hb) (15, 50), SLT-IIc (51), and several others (43, 66). SLT-IIc, SLT-IIvha, and SLT-IIvhh are 97% homologous to SLT-II at the deduced amino acid sequence level (15, 51) but are antigenically distinct from SLT-II (14, 28, 51). Because of this high degree of homology, a World Health Organization subcommittee on SLT/VT nomenclature recently recommended that these SLT-II-related toxins all be designated as SLT-IIc (subcommittee meeting held at the conference "Shiga-Like Toxin Producing *Escherichia coli* with a Special Emphasis on Zoonotic Aspects," 9 to 12 December 1991, Giessen, Germany). Therefore, SLT-IIvha, SLT-IIvhh, SLT-IIc, and all other SLT-II-related toxins that exhibit only partial serological cross-reactivity to SLT-II will be referred to as SLT-IIc in this article.

To study the pathogenesis of EHEC infection, we previously developed an orally infected, streptomycin-treated mouse model. Mice fed O157:H7 EHEC strain 933cu-rev died and displayed acute bilateral renal tubular necrosis (61). Kidney damage in these orally infected mice appeared to be SLT mediated (56, 62). In a subsequent investigation, we demonstrated that SLT-II, not SLT-I, is responsible for the virulence of strain 933cu-rev in the orally infected, streptomycin-treated mouse model (62). In the studies reported here, we used the orally infected mouse model to examine the virulence of four multi-SLT-II-producing clinical EHEC strains.

(A portion of this work was presented at the 92nd General Meeting of the American Society for Microbiology, New Orleans, La., 26 to 30 May 1992 [28].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Tables 1 and 2, respectively. For the in vivo studies, a spontaneous strepto-

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TABLE 1. *E. coli* strains used in this study

Strain	Serotype	No. of <i>slt</i> genes	SLT type	Reference
EHEC strains^a				
B2F1	O91:H21	2	IIc ^b	15
H414-36/89	O91:H21	3	II, IIc	This study
J-2	O157:H7	2	II, IIc	66
933	O157:H7	2	I, II	54
E32511	O157:H ⁻	2	II, IIc	51
E32511/HSC ^c	O157:H ⁻	1	IIc	13
Other <i>E. coli</i> strains				
C600	Rough	0	NA ^d	NA
DH5 α	Rough	0	NA	NA

^a The following individuals kindly provided the EHEC strains used in this study: M. Karmali, B2F1 and E32511/HSC; S. Aleksic, H414-36/89; Y. Takeda, J-2; I. K. Wachsmuth, 933; B. Rowe, E32511.

^b The designation IIc includes the SLT-II-related toxins originally called SLT-IIvha, SLT-IIvha, and SLT-IIc.

^c E32511/HSC is a spontaneously derived mutant of E32511 that appears to have lost the phage that encodes SLT-II (13).

^d NA, not applicable.

mycin-resistant (Str^r) mutant was derived from each of the clinical EHEC isolates. The in vitro growth rates and levels of cytotoxicity for each of the Str^r derivatives were found to be comparable to those of the parental strain.

Media, enzymes, and biochemicals. Bacteria were routinely cultured in Luria-Bertani (LB) medium (30). Clinical EHEC strains were plated on MacConkey agar (Difco, Detroit, Mich.). Where indicated or required for selective pressure, media were supplemented with antibiotics (Sigma Chemical Co., St. Louis, Mo.) as follows: ampicillin, 200 μ g/ml; streptomycin, 30 μ g/ml; nalidixic acid, 50 μ g/ml; rifampin, 50 μ g/ml. Restriction enzymes, RNase, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Fetal calf serum (silver grade) was obtained from ICN Biomedicals, Inc. (Irvine, Calif.), and Eagles minimum essential medium was purchased from Whittaker Bioproducts, Inc. (Walkersville, Md.).

Mouse feeding, colonization, and oral LD₅₀ studies. The method for mouse feeding was essentially as described previously (61, 62). Five- to six-week-old male CD-1 outbred mice (Charles River Laboratories, Wilmington, Mass.) were used for all of the in vivo experiments in this study.

TABLE 2. Plasmids used in this study

Plasmid	Toxin produced ^a	Description
pJES120 ^b	SLT-II	2.4-kb <i>Bam</i> HI- <i>Eco</i> RI fragment from pLMS2.2 (55) in pSK(-) creating a promoterless <i>slt</i> -II under the direction of the β -galactosidase promoter
pSQ12	SLT-IIvha	<i>slt</i> -IIvha from B2F1 in pHC79 ^c
pSQ343	SLT-IIvha	2.3-kb <i>Pst</i> I- <i>Eco</i> RV fragment from pSQ12 in pKS(-)
pSQ47	SLT-IIc	<i>slt</i> -IIc from H414-36/89 in pSK(-)
pSQ81	SLT-II	<i>slt</i> -II from H414-36/89 in pSK(-)
pSQ135	SLT-IIc	<i>slt</i> -IIc from H414-36/89 in pSK(-)

^a *slt*-IIvha, *slt*-IIvha, and *slt*-IIc and the corresponding protein products are referred to as *slt*-IIc and SLT-IIc in this article.

^b Constructed and kindly provided by J. E. Samuel.

^c Construction of the strain B2F1 cosmid bank in pHC79 was previously described (50).

Throughout each experiment mice were given water that contained streptomycin (5 g/liter). Streptomycin treatment reduces the level of facultative anaerobic bacteria that normally colonize the mouse intestine (36). Intestinal colonization by the test organism was determined by the persistence of bacterial shedding in feces over time, as previously described (61). If all animals in a group fed 10¹⁰ CFU died, groups of five animals were fed graded doses of the organism to assess the oral 50% lethal dose (LD₅₀). The initial 10¹⁰-CFU suspension was diluted in 20% sucrose to yield the dose required in a 50- μ l sample. Each 50- μ l aliquot was fed to mice with a Pipetman (Ranin, Woburn, Mass.). A sample of each bacterial suspension was diluted and plated on selective agar to assess the actual CFU fed. The dose of bacteria required to kill 50% of the mice was calculated by the method of Reed and Muench (47). The experiments reported here were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (37).

Histological studies. Mouse tissues were isolated from moribund mice or healthy controls. The tissue specimens examined included but were not limited to brain, heart, lungs, trachea, liver, kidneys, spleen, adrenal glands, pancreas, submandibular lymph nodes, esophagus, stomach, small intestine, cecum, and colon. The specimens were fixed in 10% buffered formalin, processed routinely for histologic examination, and stained with hematoxylin and eosin. All histological sections were coded and assessed for histopathologic changes by light microscopy.

Hematological analyses. Blood was obtained from the carotid arteries of experimental mice. Whole blood was assessed for hemoglobin concentration, hematocrit, and platelet count with a Baker 9000 cell counter (Serono Baker Diagnostics, Allentown, Pa.). Serum was isolated from whole blood and tested for concentration of blood urea nitrogen (BUN), creatinine, and total protein with the Baker Centrifchem System 500 chemistry analyzer (Serono Baker Diagnostics). The reagents for the blood chemistry analysis were purchased from Trace America (Miami, Fla.).

Passive immunization experiments. Mice were injected intraperitoneally with 1 ml of diluted ascites fluid containing either immunoglobulin G monoclonal anti-SLT-II B subunit antibody (designated BC5BB12 [6]) or a control immunoglobulin G monoclonal anti-SLT-I B subunit antibody (designated 13C4 [53]). The injections were given to groups of 5 to 10 mice 1 day before and again immediately prior to oral inoculation with *E. coli* O91:H21 strain B2F1(Str^r) or H414-36/89(Str^r).

Identification of EHEC colonization sites. The location at which a particular EHEC strain colonized the intestine of streptomycin-treated mice was evaluated as follows. Groups of four streptomycin-treated mice were fed 10¹⁰ CFU of each *E. coli* strain to be tested. The animals were sacrificed when moribund or 18 to 24 h prior to the time of death estimated on the basis of preliminary studies. Intestinal segments of 3 cm were excised from the proximal, mid, and distal small intestine; the cecum; and the proximal and distal colon of each mouse. The tubular segments were then washed to remove fecal material by forcing sterile phosphate-buffered saline (PBS) through the bowel lumen with a sterile 22-gauge needle attached to a 1-ml syringe. The washed segments were then either homogenized or used to isolate epithelial cells. Epithelial cells from each 3-cm segment were prepared by the method of Weiser (64). No attempts to remove bacteria from the epithelial cells prior to plating were made. The homogenized intestinal segments and the epithelial cell

suspensions were diluted in PBS and plated on MacConkey agar containing streptomycin to determine the number of CFU per intestinal segment.

Growth of EHEC strains in crude intestinal mucus. The growth of *E. coli* in mouse intestinal mucus was assessed as described previously (61). Mucus was isolated from 5-cm intestinal segments of mouse proximal, mid, and distal small intestine and the proximal and distal colon.

DNA hybridization studies. Total DNA was isolated as described in *Current Protocols in Molecular Biology* (1) and digested with either *EcoRI* or *HindIII*, and the fragments were separated by agarose (0.9%) gel electrophoresis. The agarose gels were then probed by in situ gel hybridization and washed under stringent conditions as detailed elsewhere (51). The *slt-II* probe used was a 1-kb *SmaI-ScaI slt-II*vha (now called *slt-IIc*) fragment that contains sequences from 60 bp 3' of the A subunit gene start codon to 135 bp 3' of the B subunit gene start codon. This fragment was excised from pSQ343 (Table 2) and was constructed to identify all known members of the *slt-II* toxin group. The *slt-II* DNA probe was labeled with [³²P]dCTP (New England Nuclear Research Products, Boston, Mass.) by nick translation (BRL Nick Translation System; Life Technologies, Gaithersburg, Md.).

Cloning toxin genes from EHEC strain H414-36/89. In situ gel hybridization of *EcoRI*-digested H414-36/89 DNA identified fragments of 4.1, 8.4, and 12.5 kb that contained *slt-II* or an *slt-II*-related toxin gene. Total H414-36/89 DNA was digested with *EcoRI* and separated by agarose (0.7%) gel electrophoresis. Regions of the agarose gel corresponding to DNA of 4 to 5, 8 to 9, and 12 to 14 kb were isolated. DNA was purified from each of the gel slices by using the GeneClean system (Bio 101, La Jolla, Calif.) and then amplified by polymerase chain reaction (PCR) with either *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) or Vent polymerase (New England Biolabs, Beverly, Mass.) in a thermocycler (Perkin-Elmer Cetus). Primers of 21 and 25 bp were used for the amplification; these primers were designed to anneal at approximately 322 bp 5' to the *slt-II* A subunit gene start codon and 58 bp 3' to the *slt-II* B subunit gene stop codon, respectively. Amplification with these primers generated unique *Sau3A* and *EcoRI* sites 5' and 3' to the toxin gene, respectively. The PCR products were isolated, purified by using the GeneClean system (Bio 101), digested with both *Sau3A* and *EcoRI*, and ligated into *BamHI-EcoRI*-digested Bluescript vector pSK(-) (Stratagene). Toxin-producing DH5 α transformants were identified by Vero cell cytotoxicity assays.

Bacteriophage induction, plaquing, and lysogen isolation. Bacteriophage were released by mitomycin induction (34). LB cultures of H414-36/89 were grown at 37°C with agitation. Mitomycin was added (final concentration of 1 μ g/ml) when the bacterial culture reached an optical density at 600 nm of 0.300. After overnight incubation at 37°C with vigorous agitation, the lysed bacterial culture was centrifuged to remove cellular debris and the supernatant was filter sterilized. For phage plaquing, 0.1 ml of the EHEC ϕ H414 lysate was incubated with an equal volume of an exponential-phase culture of *E. coli* C600 for 20 min at 37°C. Soft agar was then added, and the mixture was plated on petri dishes that contained hard agar. Lysogens of ϕ H414 were purified from colonies at the peripheries of three purified plaques. LB broth and agar supplemented with 10 mM CaCl₂ were used throughout all the phage experiments.

Cytotoxicity and immunoreactivity assays. Samples of bacterial culture supernatants or sonically disrupted bacterial cell pellets were tested for cytotoxicity on Vero cells by the

method of Gentry and Dalrymple (10) as modified by Schmitt et al. (51). The cytotoxic titer was expressed as the reciprocal of the highest dilution required for 50% cell death per milliliter of culture (CD₅₀/ml). Toxin immunoreactivity assays were done by incubating a single dilution of antibody with serial twofold dilutions of toxin for 4 h at 37°C. The toxin-antibody mixtures were then added to Vero cells. The antibodies and dilutions used for toxin immunoreactivity experiments were as follows: polyclonal anti-SLT-II (designated AJ65 [54]) at a 1:50 dilution; monoclonal anti-SLT-II A subunit (designated 2E1 [44]), neat; ascites of monoclonal anti-SLT-II B subunit (designated BC5BB12 [6]) at a 1:100 dilution.

RESULTS

Virulence and colonization capacity of SLT-II-producing *E. coli* clinical isolates in mice. In an earlier study from this laboratory, Wadolkowski et al. observed that EHEC O157:H7 strain 933, which makes SLT-I and SLT-II (54), does not kill orally challenged, streptomycin-treated mice (61). However, in that study, a variant of a plasmid-cured derivative of strain 933 (933cu-rev) that is lethal for streptomycin-treated mice at about 10¹⁰ CFU was isolated (61). Subsequent experiments revealed that SLT-II but not SLT-I production by 933cu-rev was responsible for the death of streptomycin-treated, orally infected mice (62). To assess the virulence of other EHEC isolates in this model and to verify the importance of SLT-II production in virulence, we tested several clinical isolates that contained at least one toxin gene from the SLT-II group. Mice were treated with streptomycin and individually fed 10¹⁰ CFU of Str^r derivatives of O91:H21 strain B2F1, O91:H21 strain H414-36/89, O157:H7 strain J-2, O157:H⁻ strain E32511 and its derivative E32511/HSC, and O157:H7 strain 933 (as an avirulent control).

Mice fed the Str^r derivatives were monitored for intestinal colonization (Fig. 1) and death (Table 3). Mice in every test group were colonized (Fig. 1). Strain 933 was previously shown to colonize CD-1 mice (see Fig. 1 in reference 61). All of the mice fed B2F1(Str^r) or H414-36/89(Str^r) died between days 3 and 5 postfeeding (Table 3); therefore, strains B2F1 and H414-36/89 were classified as highly virulent in this mouse model. A few mice fed strain E32511(Str^r) or E32511/HSC(Str^r) died between days 5 and 23 postfeeding (Table 3). Passage of strains E32511(Str^r) and E32511/HSC(Str^r) through mice did not increase the pathogenicity of these isolates (data not shown). One mouse of 19 fed strain J-2(Str^r) died, and strain 933(Str^r) was avirulent in the mouse model (Table 3).

Next, we sought to determine whether streptomycin treatment was required for colonization and virulence of B2F1(Str^r) or H414-36/89(Str^r). To address this question, a dose of 10¹⁰ CFU of the highly virulent strain B2F1(Str^r) or H414-36/89(Str^r) was fed to non-streptomycin-treated mice. By day 4 postinoculation, the number of CFU per gram of feces of mice fed either of these O91:H21 strains had dropped to approximately 5 $\times$ 10⁴ CFU/g of feces or lower (Fig. 2). Furthermore, by day 8 postinfection, both EHEC strains were present in the feces close to or below the level of detection (10² CFU/g of feces), and none of the mice in either group died. These data suggest that suppression of normal intestinal flora is required for high-level colonization and subsequent death of mice fed EHEC O91:H21 strain B2F1(Str^r) or H414-36/89(Str^r).

To assess the oral LD₅₀s of the highly virulent strains,

FIG. 1. Pattern of colonization of streptomycin-treated mice fed Str^r derivatives of EHEC O91:H21 strain B2F1 or H414-36/89 (A) or O157:H⁻ strain E32511 or E32511/HSC or O157:H7 strain J-2 (B). At the times indicated, fecal samples were isolated from individually housed mice, homogenized, and plated on MacConkey agar that contained streptomycin. Each point represents the geometric mean CFU per gram of feces from three mice from a single experiment. Colonization experiments were done one to five times for each strain. All of the mice fed B2F1(Str^r) or H414-36/89(Str^r) died by day 4 postfeeding. Because each datum point represents three mice, error bars are not presented.

groups of five streptomycin-treated mice were fed dilutions of 10^1 to 10^{10} CFU of either B2F1(Str^r) or H414-36/89(Str^r). At least four of five mice from each group fed the various doses of B2F1(Str^r) died, and five of five mice from each of the H414-36/89(Str^r) groups died. Therefore, the oral LD_{50} s for both B2F1(Str^r) and H414-36/89(Str^r) were less than 10^1 CFU (Table 3). Colonization data from streptomycin-treated mice fed 10^1 CFU of strain B2F1(Str^r) or H414-36/89(Str^r) indicated that even when fed low doses, the mice were rapidly colonized at levels of 10^8 CFU/g of feces (data not shown). These animals died within 5 to 8 days postfeeding.

Histological examination of moribund mice. To determine

the cause of death of the mice infected with EHEC strain B2F1(Str^r), moribund animals were sacrificed and a variety of tissues were examined histologically. No lesions were observed in any sections except those from the kidneys, in which dilation, degeneration, and necrosis of renal tubules were observed. Extensive renal tubular necrosis was also seen in the kidneys of moribund mice infected with *E. coli* H414-36/89(Str^r) (data not shown). These results suggest that the mice fed either B2F1(Str^r) or H414-36/89(Str^r) died of renal tubular necrosis, a supposition consistent with previous histological findings from mice fed 933cu-rev (see Fig. 6 in reference 61).

TABLE 3. Virulence of EHEC strains for orally inoculated, streptomycin-treated mice

EHEC strain fed	No. of mice that died/no. inoculated ^a	Oral LD ₅₀ ^b (CFU)
B2F1(Str ^r)	21/21	<10
H414-36/89(Str ^r)	9/9	<10
E32511(Str ^r)	3/23	ND ^c
E32511/HSC(Str ^r)	4/19	ND
J-2(Str ^r)	1/19	ND
933(Str ^r)	0/19	ND

^a Mice were orally inoculated with 10¹⁰ CFU of bacteria. Each ratio represents pooled data from three to six separate experiments.

^b The LD₅₀ was determined by the method of Reed and Muench (47).

^c ND, not done because few or no mice in these groups died at oral doses of 10¹⁰ CFU.

Hematological examination of mice infected with O91:H21 strain B2F1. As mentioned previously, some patients infected with EHEC develop HUS. Hallmarks of HUS include renal glomerular damage, thrombocytopenia, and hemolytic anemia (22). The severity of HUS is assessed by renal function tests, blood platelet count, and blood hemoglobin concentration. In this study, hematological and serum biochemical test results were obtained from B2F1(Str^r)-infected and uninfected control mice to ascertain whether the moribund mice had developed clinical HUS (Table 4). Although the blood chemistry and hematological results obtained were variable, slight increases in hemoglobin, hematocrit, and

platelet counts were noted. The hematological test results obtained were contradictory to characteristic test results from patients with HUS and may indicate hemoconcentration. The BUN and creatinine values were elevated compared with those of control mice, a finding which is consistent with renal tubular damage. However, interpretation of these kidney function test results is confounded by the possibility of hemoconcentration.

Passive protection with monoclonal antibody to SLT-II. To assess the importance of B2F1 and H414-36/89 toxin production in the mortality of infected mice, the animals were given monoclonal anti-SLT-II B subunit antibody intraperitoneally prior to oral inoculation with strain B2F1(Str^r) or H414-36/89(Str^r). Administration of anti-SLT-II B subunit antibody did not appear to affect bacterial colonization (data not shown) but did protect the mice from the lethal effects of both B2F1(Str^r) and H414-26/89(Str^r) (Table 5). By contrast, neither the control anti-SLT-I B subunit monoclonal antibody nor the PBS control prevented the deaths of the infected mice (Table 5). Furthermore, no histological damage was observed in selected anti-SLT-II B subunit antibody-treated, infected mice. Taken together, these results indicate that SLT-II plays a pivotal role in the deaths of streptomycin-treated mice fed EHEC O91:H21 strains.

In vivo colonization characteristics of strains B2F1(Str^r) and H414-36/89(Str^r). Although all of the strains examined for mouse lethality in this study produce at least one toxin from the SLT-II group, only two of the isolates were consistently lethal for mice. These data suggest that the highly virulent

FIG. 2. Pattern of intestinal colonization of non-streptomycin-treated mice by EHEC strains. At the times indicated, fecal samples were plated on MacConkey agar containing streptomycin. The bacterial detection limit for assessment of intestinal colonization was 10² CFU/g of feces. Each point represents the geometric mean CFU per gram of feces from four mice. The error bars represent ± 2 standard errors of the geometric means.

TABLE 4. Hematology and blood chemistry results of B2F1(Str^r)-infected and uninfected streptomycin-treated CD-1 mice

Mouse group	Test result ^a				
	Hemoglobin (g/dl)	Platelets (mm ³) (10 ³)	Hematocrit (%)	BUN (mg/dl)	Creatinine (mg/dl)
Streptomycin treated and B2F1(Str ^r) inoculated ^b	18.5 ± 0.6	2,238 ± 707	53 ± 4	79 ± 33	0.7 ± 0.6
Streptomycin treated and uninfected	14.6 ± 1.0	1,489 ± 174	43 ± 2	34 ± 6	0.2 ± 0.1
Untreated and uninfected ^c	14.4 ± 0.9	1,200 ± 100	42 ± 3	33 ± 5	0.5 ± 0.2

^a Values given are means ± standard deviations; *n* = 6.^b Mice were orally inoculated with 10⁶ CFU of B2F1(Str^r). Whole blood or sera from the moribund mice were analyzed as appropriate.^c Hematological values given were obtained from Charles River for 6- to 8-week-old CD-1 mice (*n* = 20).

strains B2F1 and H414-36/89 either may express more toxin *in vivo* than the other strains examined or may have additional virulence factors that augment toxin delivery to the mouse. To determine whether B2F1(Str^r) and H414-36/89(Str^r) are able to colonize the mouse intestine better than the other EHEC strains tested, we examined several parameters of colonization. First, we compared the capacities of selected strains to individually colonize the mouse intestine in the presence of normal flora. The data shown in Fig. 2 indicate that strains 933(Str^r) and E32511/HSC(Str^r) were as efficient as B2F1(Str^r) and H414-36/89(Str^r) in colonizing the normal mouse intestine. Second, we examined the capacity of the lethal EHEC strain B2F1 to compete with strain E32511 for intestinal colonization. When 10¹⁰ CFU of strain B2F1(Str^r Nal^r) (resistant to streptomycin and nalidixic acid) and 10¹⁰ CFU of strain E32511(Str^r Rif^r) (resistant to streptomycin and rifampin) were fed simultaneously to mice, the strains both persisted at levels of 10⁸ to 10⁹ CFU/g of feces until day 4, when all of the mice died (Fig. 3). These cocolonization results suggest that either the two strains compete equally well for the same colonization sites or they colonize equally well but at different locations in the mouse intestine. The third aspect of colonization we investigated was whether B2F1(Str^r) or H414-36/89(Str^r) colonized the mouse small intestine. It was previously shown that the mouse-virulent EHEC strain 933cu-rev, unlike its mouse-avirulent parent 933cu or 933, could colonize the small as well as the large intestine at high levels (61). In the experiments described here, B2F1(Str^r) and H414-36/89(Str^r) were occasionally, but not consistently, found in high numbers in the small intestine (data not shown). The inconsistency of bacterial counts in the small intestinal segments may have been due, in part, to experimental manipulations. If the bacteria actively grew in the lumen and did not firmly adhere

to the intestinal epithelial cells, the organisms could have been removed by the initial washing of the 3-cm segments.

As a corollary to the intestinal colonization studies, we assessed whether the EHEC strains could grow in intestinal mucus *in vitro* and, if so, whether there was a relationship between extent of growth and virulence. Approximately 10³ CFU of strain B2F1(Str^r), H414-36/89(Str^r), or E32511(Str^r) or avirulent strain 933(Str^r) was inoculated into crude mucus preparations and allowed to grow at 37°C for 18 to 24 h. The geometric mean CFU per milliliter of culture from pooled experimental results for each strain is presented in Fig. 4. Strains B2F1(Str^r), H414-36/89(Str^r), and E32511(Str^r) grew to levels of 5 × 10⁸ to 5 × 10⁹ CFU/ml in the mucus isolated from all of the small intestinal segments. By contrast, avirulent strain 933(Str^r) grew in small intestinal mucus to levels of only 1 × 10⁶ to 6 × 10⁶ CFU/ml (Fig. 4). Moreover, considerable variation in growth levels of strain 933 in proximal small intestinal mucus was observed in the nine experimental trials, as reflected by the error bar in Fig. 4. As anticipated from the work of Wadolkowski et al. (61), no differences were found in the levels of growth in large intestinal mucus among the strains tested (data not shown). Furthermore, strains B2F1(Str^r), H414-36/89(Str^r), E32511(Str^r), and 933(Str^r) all grew equally well in LB medium at 37°C (data not shown). Taken together with the *in vivo* colonization findings, the growth of the two highly virulent strains B2F1 and H414-36/89 in small intestinal mucus *in vitro* supports the hypothesis that these strains may also grow in small intestinal mucus *in vivo*. However, this *in vitro* growth characteristic is not a unique trait of the O91:H21 EHEC strains, since E32511(Str^r) also grew well in mouse small intestinal mucus *in vitro*.

SLT gene profile of strain H414-36/89. Previous DNA colony blot and toxin neutralization results identified H414-36/89 as an SLT-II producer (3). To further characterize this highly mouse-virulent strain, we sought to determine the number of *slt-II* and/or *slt-IIc* genes present. Total DNA was isolated from strain H414-36/89 as well as from strains with established toxin gene profiles. These DNA samples were digested with either *EcoRI* or *HindIII* because neither of these restriction enzymes cuts within genes from the SLT-II group (15, 17, 51, 63). *In situ* gel hybridization with an *slt-II* probe resulted in the predicted number of fragments: two for B2F1, two for E32511, and one for E32511/HSC. However, H414-36/89 had three toxin gene fragments (Fig. 5).

Cloning of H414-36/89 toxin genes and characterization of the toxin gene products by cytotoxicity and immunoreactivity assays. It was evident from the passive immunization results that SLT-II production is critical for the deaths of orally infected mice. However, it is not clear whether the *slt-II* type and toxin gene copy number contained in an EHEC strain has any bearing on its virulence in the orally infected,

TABLE 5. Results of passive immunization of streptomycin-treated CD-1 mice infected with *E. coli* O91:H21 strain B2F1(Str^r) or H414-36/89(Str^r)

Monoclonal antibody	Subunit against which the monoclonal antibody is directed	No. of mice remaining alive/no. of mice initially infected with ^a :	
		B2F1(Str ^r)	H414-36/89(Str ^r)
BC5BB12	SLT-II B	10/10	5/5
13C4	SLT-I B	1/10	0/5
None ^b		1/10	0/5

^a Mice were inoculated intraperitoneally with 1 ml of a 1:1,000 dilution of ascites fluid in PBS or PBS alone both 24 h and immediately prior to feeding 10⁶ CFU of bacteria. Experiments were done three times for B2F1(Str^r)-challenged mice and once for H414-36/89(Str^r)-challenged mice. The results presented for B2F1(Str^r) are from one experiment but are representative of all three experiments.

^b A PBS control was used.

FIG. 3. Cocolonization pattern of streptomycin-treated mice fed 10^{10} CFU each of strain B2F1 (Str^r Nal^r) and strain E32511 (Str^r Rif^r). At the times indicated, fecal samples were plated on MacConkey agar that contained streptomycin and rifampin and on MacConkey agar that contained streptomycin and nalidixic acid. Each point represents the geometric mean CFU per gram of feces from four mice. The error bars represent ± 2 standard errors of the geometric means.

streptomycin-treated mouse model. To begin to clarify these issues, the three putative H414-36/89 toxin genes were cloned from isolated *Eco*RI fragments by PCR amplification. Supernatants from DH5 α transformed with the resulting toxin clones, pSQ47, pSQ81, and pSQ135, were subjected to cytotoxicity and immunoreactivity analyses.

Two lines of evidence suggest that DH5 α (pSQ47) and DH5 α (pSQ135) produce SLT-IIc and DH5 α (pSQ81) expresses SLT-II. First, we recently observed that culture supernatants of *E. coli* K-12 strains transformed with plasmids that express SLT-IIc are 100- to 1,000-fold less cytotoxic for our Vero cells than are supernatants from similarly constructed transformants that produce SLT-II (27). Therefore, the observation that cultures of *E. coli* DH5 α supernatants transformed with pSQ47 or pSQ135 were approximately 100-fold less cytotoxic than the supernatant of DH5 α (pSQ81) suggests that pSQ47 and pSQ135 contain *slt*-IIc and pSQ81 carries *slt*-II. Second, previous studies in our laboratory have demonstrated that SLT-IIc cytotoxicity is only slightly reduced after incubation with monoclonal or polyclonal antisera raised against the SLT-II prototype (27, 28). In this study, the cytotoxicities of supernatants from DH5 α containing pSQ343 (encoding SLT-IIc), pSQ47, and pSQ135 were reduced only 10- to 100-fold after incubation with anti-SLT-II polyclonal or monoclonal antibodies (Fig. 6). Moreover, the cytotoxicities of supernatants from DH5 α containing pJES120 (encoding SLT-II) or pSQ81 decreased as much as 100,000- to 1,000-fold (depending on the antibody) after treatment with the same antibodies (Fig. 6).

These cytotoxin immunoreactivity results are consistent with the assumption that pSQ47 and pSQ135 contain *slt*-IIc and pSQ81 contains *slt*-II. It should be noted that the anti-SLT-II B subunit antibody only partially reduced SLT-IIc Vero cell cytotoxicity in vitro but this same antibody fully protected orally infected mice from death. The basis for this discrepancy is unclear. Finally, we determined that the H414-36/89 *slt*-II gene is carried on a toxin-converting phage. This conclusion was based on cytotoxicity and immunoreactivity results obtained with culture supernatants from a C600 lysogen containing a phage induced from H414-36/89 (designated ϕ H414; data not shown).

DISCUSSION

In this study, several EHEC strains that produce SLT-II and/or SLT-IIc were fed to streptomycin-treated mice. Two of the strains tested, O91:H21 strain B2F1 (isolated from a Canadian patient with HUS) and O91:H21 strain H414-36/89 (isolated from a German patient with hemorrhagic colitis), had LD₅₀s of <10 organisms. Histopathologic examination of kidneys from the moribund mice fed O91:H21 strain B2F1 revealed degeneration and necrosis of renal tubules. This pattern of kidney damage is consistent with that observed in mice fed EHEC strain 933cu-rev (61). However, these morphological changes differ from the renal glomerular destruction reported for human HUS. This species-specific difference in the type of renal cells damaged appears to be a consequence of variation in toxin receptor distribution in the

Small intestinal region

FIG. 4. Growth of EHEC strains in small intestinal mucus. Crude mucus was inoculated with 10^3 CFU of bacteria. After incubation at 37°C for 19 h, samples were diluted and plated on MacConkey agar containing streptomycin. Each bar represents the geometric mean CFU from four to nine experiments. The error bars represent +2 standard errors of the means.

kidney (56). Blood chemistry findings from B2F1-infected mice also deviated from typical laboratory results observed for human HUS. Patients with HUS have renal glomerular damage, thrombocytopenia, and hemolytic anemia (24). The B2F1-infected mice had slight increases in hemoglobin, hematocrit, and platelet counts, findings which suggest hemoconcentration. Because hemoconcentration occurs frequently in moribund animals (4a), we were not surprised by this observation. Furthermore, it has been hypothesized that the hemolytic anemia of HUS occurs when erythrocytes are ruptured as they pass through damaged renal glomeruli (4, 12). If this hypothesis is true, hemolytic anemia would not occur in the EHEC-infected moribund mice because renal tubules, not vascular glomerular cells, are damaged in these animals. Although the BUN and creatinine values in the infected mice were increased, the hemoconcentration effects obscure the interpretation of these renal function results. On the basis of the histopathology and hematology data, we conclude that the streptomycin-treated mouse model is not a model for HUS but is useful as a tool for assessing the virulence of orally administered EHEC.

The virulence of strains B2F1(Str^r) and H414-36/89(Str^r)

FIG. 5. Copies of *slt-II* genes in EHEC clinical isolates B2F1, H414-36/89, E32511, and E32511/HSC. Total DNA was digested with *Eco*RI (A) or *Hind*III (B) and hybridized with a 1-kb *Sma*I-*Sca*I *slt-II*vha probe by in situ gel hybridization. Numbers to the left are marker DNA fragments in kilobase pairs.

was directly correlated with SLT-II and/or SLT-IIc production by the O91:H21 EHEC strains, as demonstrated by passive immunization studies. However, the other EHEC strains examined produced at least one toxin from the SLT-II group but were not as virulent as the O91:H21 isolates. Thus, the mere presence of *slt-II* or *slt-IIc* in an EHEC strain does not guarantee virulence for mice, nor does it appear to matter which of the SLT-II subtypes is produced by an EHEC isolate. In support of the latter conclusion, we recently found that purified SLT-II and SLT-IIc have the same LD₅₀ for parentally injected mice (27).

One possible explanation for the marked virulence of the O91:H21 isolates is that these EHEC strains are particularly efficient at systemic delivery of toxin from the gut. Because the site at which EHEC strains colonize the intestine of streptomycin-treated mice and the mechanism by which the bacteria adhere could affect delivery of toxin, we evaluated several facets of EHEC O91:H21 strain-intestine interaction. We found that the two O91:H21 virulent strains could colonize the mouse small intestine, a region that may facilitate significant systemic absorption of SLT. However, we could not consistently demonstrate colonization at this site. The variable small bowel colonization data suggested to us that EHEC O91:H21 strains might grow to high numbers at that intestinal location but may not adhere avidly to the epithelial cell surface. Indeed, we determined that the two O91:H21 strains grew readily in small intestinal mucus. Though this growth characteristic may enhance the systemic delivery of toxin, it was not limited to the O91:H21 strains; strain E32511(Str^r), which killed only 3 of 23 mice fed 10^{10} organisms, also grew well in small intestinal mucus. These data suggest that the capacity of B2F1 and H414-36/89 to grow in mucus isolated from the small intestine may contribute to the virulence of these strains but that another component(s) must also be involved since E32511 was less virulent in the model.

One difference between the O91:H21 strains and the other EHEC strains tested is that the O91:H21 strains do not contain the *eae* gene which has been suggested to be involved in adherence of prototypic EHEC strains (58).

FIG. 6. Residual cytotoxicity of EHEC strain H414-36/89 toxins after incubation with various antisera (immunoreactivity profile). The bars indicate the levels of Vero cell cytotoxicity of culture supernatants or sonically disrupted bacterial cell pellets from DH5 α containing pJES120 (*slt*-II), pSQ343 (*slt*-IIc), pSQ47 (*slt*-IIc), pSQ81 (*slt*-II), or pSQ135 (*slt*-IIc). Samples were incubated in the absence or presence of the following antibodies: polyclonal anti-SLT-II, monoclonal anti-SLT-II A subunit, or monoclonal anti-SLT-II B subunit.

Total DNA from O91:H21 strains B2F1 and H414-36/89 did not hybridize with an *eae* gene probe (18) even under low-stringency conditions (35). That the O91:H21 strains may adhere to epithelial cells by a mechanism distinct from prototypic *eae*-probe-positive EHEC could, in part or wholly, explain the particularly pathogenic phenotype of these strains for streptomycin-treated mice.

During the course of this study, we discovered that strain H414-36/89 contained three *slt*-II genes. Recently, several laboratories have identified EHEC strains that carry two toxin genes from the SLT-II group (13, 51, 59, 66). To our knowledge, this is the first definitive report of a clinical isolate that contains three toxin genes from the SLT-II group. Two of the H414-36/89 toxins expressed from genes cloned from this strain had a toxin immunoreactivity profile similar to that of SLT-IIc. The gel migration pattern of the 4.1-kb *Eco*RI fragment containing one of these genes and that of the *Eco*RI fragment containing the *slt*-IIvhb (now called *slt*-IIc) gene from B2F1 were indistinguishable. Similarly, the size of one of the *slt*-II probe-positive *Hind*III (Fig. 5), *Pst*I, and *Bam*HI (data not shown) fragments of H414-36/89 DNA was nearly identical to that of the *slt*-IIvhb fragment of B2F1. Although the nucleotide sequences of the *slt* genes from H414-36/89 were not determined, the immunoreactivity and hybridization results support the tenet that this strain carries one copy of *slt*-II and two copies of *slt*-IIc. Moreover, in contrast to results with B2F1, which does not carry *slt*-II, we were able to induce an SLT-II toxin-converting phase from strain H414-36/89.

The striking virulence of strains B2F1 and H414-36/89 is

particularly troublesome in light of the observation that neither strain would be detected by techniques currently used in many clinical laboratories in the United States. These methods of detecting EHEC focus on the O157:H7 or O157:H⁻ EHEC serotype and the observation that many O157:H7 and O157:H⁻ strains do not ferment sorbitol (31). EHEC strains H414-36/89 and B2F1 are of the O91:H21 serotype and ferment sorbitol. Consequently, without screening for toxin production or the presence of toxin genes, neither of these strains would have been identified as the causative agents of hemorrhagic colitis and HUS. Clinical laboratories could achieve a more frequent and accurate identification of an EHEC infection if stool samples or *E. coli* isolates from stools were screened for *slt* genes. Recent epidemiological studies have recommended this approach (3). To facilitate such identification, *slt* probes have been generated (8, 13, 21, 38, 59) and procedures for identifying *slt* genes from isolated colonies using PCR technology have been developed (13, 16, 32, 45, 59).

In conclusion, we have identified two clinical EHEC isolates that are highly pathogenic for orally infected, streptomycin-treated mice. Our studies indicate that the deaths of these mice are due to the production of SLT-II/IIc, but the production of toxin alone is insufficient to explain why these particular isolates are so virulent in this model. Perhaps strains B2F1 and H414-36/89 produce more toxin than the other EHEC strains *in vivo*, deliver toxin more effectively, or, conceivably, adhere differently from the *eae* probe-positive strains.

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