CHARACTERIZATION AND VIRULENCE ASSESSMENT OF TWO O91:H21 ENTEROHEMORRHAGIC *ESCHERICHIA COLI* ISOLATES

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ABSTRACT

Title of Dissertation:

Characterization and Virulence Assessment of Two 091:H21 Enterohemorrhagic *Escherichia coli* Isolates

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Enterohemorrhagic *Escherichia coli* (EHEC) cause food-borne hemorrhagic colitis and the hemolytic uremic syndrome. EHEC produce Shiga-like toxin(s) (SLTs) type I (SLT-I) or type II (SLT-II) or both. SLTs consist of one A subunit and five B subunits and cause inhibition of protein synthesis and subsequent cell death. *E. coli* K-12 strains which produce high levels of SLT-II but not SLT-I were previously shown to be virulent in an orally-infected, streptomycin-treated mouse model. In this investigation, the pathogenicity of several SLT-II-producing EHEC isolates was compared. All of the strains tested colonized the mouse intestine. However, only two strains were consistently virulent for mice: 091:H21 strain B2F1(Str^r) which carries two copies of *slt*-II-related

toxins, *slt*-IIvha and *slt*-IIvhb, and 091:H21 strain H414-36/89(Str^r) which was found in this study to contain three genes from the *slt*-II toxin group. The oral LD_{50} of strains B2F1(Str^r) and H414-36/89(Str^r) when fed to streptomycin-treated mice was less than 10 bacteria. Histological sections from moribund mice fed the 091: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 091:H21 EHEC strains was supported by the finding that streptomycin-treated mice pre-inoculated 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 striking virulence of the 091:H21 strains among SLT-II-producing EHEC strains was not determined, but intestinal colonization studies suggested that delivery of toxin *in vivo* might be particularly efficient. The type of SLT-II produced by an EHEC strain did not appear to affect virulence because purified SLT-IIvhb and SLT-II had comparable *in vivo* 50% lethal doses. However, SLT-IIvhb was 500-fold less toxic for Vero cells. The lower cytotoxic activity of SLT-IIvhb appears to be due to a charge difference at amino acid position 16 in the receptor binding portion of the B subunit.

Characterization and Virulence Assessment of Two 091:H21 Enterohemorrhagic *Escherichia coli* Isolates

by

Susanne W. Lindgren

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To Rob,

the love of my life

and sustainer of my dreams.

"Nothing great was ever achieved without enthusiasm."

-Ralph Waldo Emerson

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INTRODUCTION

Preface

The introduction of this dissertation is divided into six sections. The first section provides a general description of pathogenic *E. coli* described to date. The second section describes the enterohemorrhagic *E. coli* (EHEC) family with a detailed description of the diseases caused by EHEC. The third section is a discussion of the Shiga-like toxins that EHEC elaborate. The fourth section presents an historical overview of the animal models that have been developed to study the pathogenesis of EHEC. The fifth section provides a description of the EHEC strains that are examined in this dissertation. The final section of the introduction outlines the specific objectives of this dissertation.

I. Overview of pathogenic *Escherichia coli* that cause diarrhea.

E. coli represent a predominant portion of the human intestinal facultative microflora. In the early 1900's researchers began to recognize that some strains of *E. coli* were pathogenic. These pathogenic *E. coli* caused both nosocomial and "summer" diarrhea, the latter of which occurred primarily in children. Over the past thirty years, a wealth of knowledge on the pathogenesis and diseases caused by virulent *E. coli* has been obtained. These *E. coli* are

currently classified into five groups (Table 1). The classification scheme is based on distinct clinical manifestations, mechanisms of pathogenesis, enterotoxin production, and the O:H serotype of the diarrheagenic *E. coli* (Levine 1987). The five groups include: Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), Enteropathogenic *E. coli* (EPEC), and Enterohemmorhagic *E. coli* (EHEC) (Levine *et al.*, 1987; Vial *et al.*, 1988).

ETEC are one of the most common causes of traveler's diarrhea (Dupont *et al.*, 1976; Merson *et al.*, 1976) and have also been implicated in developing countries as a cause of infant diarrhea and dehydration (Black *et al.*, 1982; Guerrant *et al.*, 1983). ETEC colonize the proximal small intestine and elaborate heat-labile toxin (LT) and/or heat-stable toxin (ST). ETEC cause profuse watery diarrhea similar to the diarrhea caused by *Vibrio cholerae* infection (Sack, 1975).

EIEC, unlike ETEC, do not elaborate a toxin but invade and replicate within intestinal epithelial cells of the colonic mucosa. This invasive capacity, similar to *Shigella* spp., results in the eventual destruction and death of the infected cells (Tulloch *et al.*, 1973). EIEC illness is characterized by fever, severe abdominal pain, watery diarrhea, and dysentery.

Footnotes for Table 1

[a] CFA, colonization factor antigen; CS, coli surface-associated antigen; N/D, not determined; att-eff, attaching and effacing.

[b] LT, heat-labile toxin; ST, heat-stable toxin; EAST, Enteroaggregative heat-stable toxin; SLT, Shiga-like toxin.

[c] EIEC are invasive as assessed by the Sereny test. EPEC are not Sereny test positive but do penetrate HEp-2 cells *in vitro* (Reviewed in Donnenberg *et al.*, 1992).

Table 1. Characteristics of various diarrheagenic *Escherichia coli*

E. coli group	Relevant adherence phenotype, fimbriae, or adherence genes[a]	Colonization location	Enterotoxins produced[b]	Invasive[c]
ETEC	CFA/I, CFA/II, CS1-CS6 fimbriae	small intestine	LT, ST	No
EIEC	N/D	colon	None	Yes
EAggEC	aggregative "stacked-brick"	small intestine	EAST 1	No
EPEC	localized/att-eff, *eae* gene cluster	small intestine	None	Maybe
EHEC	localized/att-eff, *eae*, plasmid-encoded fimbriae	colon	SLT	No

EAggEC have been incriminated as agents of persistent diarrhea (>14 days in duration) in infants and young children in under-developed countries (Bhan *et al.*, 1989; Wanke *et al.*, 1991). EAggEC exhibit an aggregative adherence pattern or "stacked brick" appearance on epithelial cells in both *in vitro* and in *in vivo* models (Nataro *et al.*, 1988; Vial *et al.*, 1988; Tzipori *et al.*, 1992). These bacteria possess a single 60 MDa plasmid that is required for the specialized pattern of cellular adherence (Nataro *et al.*, 1992; Vial *et al.*, 1988). Presence of the 60 MDa plasmid is also required for the production of EAST 1, an EAggEC heat-stable enterotoxin first described by Savarino *et al.* (1991).

The fourth group of pathogenic *E. coli*, EPEC, include the diarrheagenic *E. coli* first described in the early 1900's. EPEC typically cause disease in children under six months of age. For several decades, these bacteria could only be identified by serotype. No virulence traits were attributed to them. More recently, however, a distinct attaching and effacing (att-eff) histopathologic lesion has been observed in EPEC-colonized human intestines (Ulshen *et al.*, 1980; Taylor *et al.*, 1986). This att-eff lesion has been reproduced both in tissue culture (Knutton *et al.*, 1987; Jerse *et al.*, 1990) and in an orally-inoculated gnotobiotic piglet model (Moon *et al.*, 1983; Tzipori *et al.*, 1985). Jerse *et al.* (1990) correlated the EPEC-associated tissue culture damage with the *eaeA* gene. Donnenberg and colleagues recently demonstrated

that the *eaeA* gene product, intimin, is critical for the intimate adherence of EPEC to intestinal epithelial cells (Donnenberg *et al.*, 1992). The fifth group of diarrheagenic *E. coli* described to date is the EHEC. The organisms in this group are the focus of this dissertation and are discussed in detail below.

II. Enterohemorrhagic *E. coli*.

History and epidemiology. In 1982, after a large outbreak of hemorrhagic colitis in Oregon and Michigan, a new serotype of diarrheagenic *E. coli* emerged, *E. coli* 0157:H7. This serotype along with 026:H11 and 0111:NM *E. coli* are now classified as prototypic EHEC (Levine *et al.*, 1987). Since the initial 0157:H7 *E. coli* outbreak in 1982, numerous cases of hemorrhagic colitis and the hemolytic uremic syndrome associated with this and other serotypes have occurred in the United States (Riley, *et al.*, 1983; Wells *et al.*, 1983; Remis *et al.*, 1984; Ryan *et al.*, 1986; Spika, *et al.*, 1986), Canada (Johnson, *et al.*, 1983; Laboratory Center for Disease Control, 1986), the United Kingdom (Day *et al.*, 1983; Smith *et al.*, 1987), Europe (Bockemuhl, *et al.*, 1992; Mariani-Kurkdjian, *et al.*, 1993), and Argentina (Lopez, *et al.*, 1989).

The primary mechanism of transmission of EHEC appears to be the ingestion of contaminated beef (often under-cooked hamburger meat) or raw milk products. Person-to-person spread

also occurs and accounts for some outbreaks in day care centers (Spika *et al.*, 1986; Belongia *et al.*, 1993). The most recent outbreak of *E. coli* 0157:H7 occurred in February of 1993 in the state of Washington. Approximately 500 people were affected by this virulent organism (Dean Owens, Washington State Dept. of Health, Olympia, WA, personal communication). The majority of the patients had eaten undercooked hamburgers purchased from outlets of the Jack-in-the-Box fast-food chain. Ten percent of the patients were infected by secondary transmission. Three children died. EHEC infections occur predominately in industrialized nations and in developing countries where a significant amount of beef is consumed. Argentina, a developing country with a high per capita beef consumption, has the highest incidence of EHEC-associated hemolytic uremic syndrome in the world (Lopez *et al.*, 1989).

Diseases caused by EHEC.

A) Hemorrhagic colitis. EHEC infection causes hemorrhagic colitis. This illness typically lasts from four to eight days and is characterized by severe abdominal pain with initial watery diarrhea followed by grossly bloody diarrhea without fever (reviewed in Riley, 1987). Later in infection, some patients develop extra-intestinal sequelae in the form of the hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (Karmali, 1989). These sequelae are characterized by

renal failure and may lead to death. The hemorrhagic colitis caused by EHEC is usually self-limiting, and antibiotic treatment is contraindicated (Ward *et al.*, 1990; Tarr, *et al.*, 1988; Sack, *et al.* 1987; Carter, *et al.* 1987). Trimethoprim-sulfamethoxazole, an antibiotic commonly given to patients with signs of diarrheal disease, has been shown to increase Shiga-like toxin production by EHEC *in vitro* (Karch *et al.*, 1986). Some researchers believe that treatment with trimethoprim-sulfamethoxazole may have the same effect on EHEC *in vivo* and may therefore increase the likelihood of toxin-mediated HUS sequelae in patients infected with EHEC (Tarr, *et al.*, 1988).

B) The hemolytic uremic syndrome. HUS is characterized by a triad of features including thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure (Levin *et al.*, 1989). HUS occurs predominantly in children under four years of age and is a common cause of renal failure in this age group (Counahan *et al.*, 1977). HUS lesions are found primarily in the vascular endothelial cells of the kidney, particularly in the renal glomeruli. The renal glomerular damage includes swollen and detached endothelial cells, as well as thrombi and fibrin deposition in the subendothelial space (Remuzzi *et al.*, 1985). EHEC-associated diarrhea was only recently identified as one of the prodromes of HUS, and

the mechanism of development of EHEC HUS is still only partially understood.

EHEC-associated virulence factors. There are three main virulence-associated characteristics of prototypic EHEC. First, they harbor a plasmid of approximately 60 MDa in size that is similar but not necessarily identical among all EHEC strains (Barrett *et al.*, 1992). The role(s) of this large plasmid(s) in virulence and/or adherence remains to be determined; data from *in vitro* and *in vivo* studies that describe a role for the plasmid in adherence are conflicting (Karch *et al.*, 1986; Junkins *et al.*, 1989; Tzipori *et al.*, 1987; Wadolkowski *et al.*, 1990a). Second, prototypic EHEC strains also contain a chromosomally located *eae* locus (Jerse *et al.*, 1990; Yu *et al.*, 1992) that, as mentioned earlier, is associated with intimate bacterial attachment to host cells by EPEC. The *eae* locus may also be important in EHEC adherence (reviewed by Tesh *et al.*, 1992). Third, EHEC strains, whether or not they are of the prototypic serotype, all produce Shiga-like toxins (SLTs), also known as Vero toxins (VTs) (for review of SLTs see Gyles *et al.*, 1992; Tesh *et al.*, 1991a; O'Brien *et al.*, 1987; Karmali *et al.*, 1985). In some clinical isolates, the *slt* operons are located on toxin-converting lambda-like lysogenic phage (O'Brien *et al.*, 1984; O'Brien *et al.*, 1989; Scotland *et al.*, 1983; Strockbine *et al.*, 1986;

Willshaw *et al.*, 1987). A more detailed discussion of SLTs will follow.

Recently, some EHEC strains have been found to produce other putative virulence factors. Johnson and co-workers described a cytolethal distending toxin (CLDT) (Johnson *et al.*, 1988, 1990). This heat-labile toxin causes progressive cell distention and is cytotoxic to sensitive cells after a 72 h period of exposure. In addition, Beutin *et al.* (1990) found an enterohemolysin which is produced by some *E. coli* serogroup O26 strains. The relationship between the CLDT or the O26 serogroup-associated enterohemolysin and *E. coli* virulence remains to be determined.

III. Shiga-like toxins.

History and biological activity. In 1977, Konowalchuk *et al.* identified a unique toxin that was produced by clinical *E. coli* isolates and cytotoxic to African green monkey kidney tissue culture cells (Vero cells) (Konowalchuk *et al.*, 1977). This group of researchers named the cytotoxin Vero toxin (VT). That same year, O'Brien *et al.* (1977) described a cytotoxin produced by certain *E. coli* isolates that was antigenically and phenotypically related to the previously identified Shiga toxin. Subsequently, O'Brien and colleagues recognized that "Shiga-like" toxin (SLT) and VT were the same protein (O'Brien *et al.* 1983b). Shiga toxin isolated from strains of

Shigella dysenteriae type 1 was originally described as a lethal toxin that causes limb paralysis when parenterally injected into mice or rabbits (Conradi *et al.*, 1903; Cavanagh *et al.*, 1956). Shiga toxin also induces fluid accumulation and epithelial cell apoptosis in ligated rabbit ileal loops (Keusch *et al.*, 1972; O'Brien *et al.*, 1980; Brown *et al.*, 1982; Eiklid *et al.*, 1983; Keenan *et al.*, 1986) and is cytotoxic for selected cells cultivated *in vitro* (Vicari *et al.*, 1960). Six years after the first publication on the similarity between Shiga toxin and SLT (O'Brien *et al.* 1982) Strockbine *et al.* (1988) demonstrated by sequence comparison that Shiga toxin and SLT type I from EHEC are virtually identical.

Mode of action and cellular targets. The enzymatic portion of Shiga toxin and the SLTs is a specific N-glycosidase that cleaves adenine residue 4324 in the 28S rRNA fragment of the eukaryotic 60S ribosomal subunit (Endo *et al.*, 1988). This depurination results in the inhibition of elongation factor 1-dependent binding of charged aminoacyl tRNA to 60S ribosomal subunits (Obrig *et al.*, 1987). Thus, these toxins inhibit protein synthesis and ultimately cause cell death. Shiga toxin and the SLTs are multi-subunit toxins that consist of one enzymatically active A subunit (approx. 33,000 MW) and five copies of a receptor-binding or B subunit (approx. 7,700 MW). These toxins are cytotoxic not only for Vero cells

but also HeLa cells (O'Brien *et al.*, 1987; Olsnes *et al.*, 1980; Gentry *et al.*, 1980). Primary human saphenous vein endothelial cells (HSVECs) and human umbilical vein endothelial cells (HUVECs) have also been used as *in vitro* models for SLT cytotoxicity (Obrig *et al.*, 1988; Tesh *et al.*, 1991b). The reason endothelial cells were chosen is because of the renal glomerular endothelial cell damage observed in patients with EHEC-associated hemolytic uremic syndrome (Karmali, *et al.*, 1985). However, these human endothelial cells are markedly less sensitive to intoxication than the established transformed cell lines (Obrig *et al.*, 1988; Tesh *et al.*, 1991b). This difference in sensitivity of primary endothelial cells and Vero cells to SLT may be due to a reduced level of functional toxin receptors in the cellular membranes (Tesh, *et al.*, 1991a).

Classification of SLTs. In recent years, two groups of SLTs have been described that differ in binding properties as well as immunological reactivity. They have been designated Shiga-like toxin type I (SLT-I) and Shiga-like toxin type II (SLT-II). The first group, SLT-I, contains the prototype toxin SLT-I and Shiga toxin. As previously mentioned, these toxins are essentially identical and will hereafter be referred to as Shiga toxin/SLT-I. Shiga toxin/SLT-I binds to the glycolipid globotriaosylceramide (Gb_3, Galα1-4Galβ1-4Glcß1-1Cer) as the functional cell-surface

receptor (Lingwood *et al.*, 1987). Polyclonal or monoclonal antibodies raised against Shiga toxin/SLT-I can neutralize the cytotoxicity of this toxin (O'Brien *et al.*, 1982).

The members of the second group, SLT-II, exhibit sequence and antigenic variation not observed in the Shiga toxin/SLT-I group. SLT-II, the prototype toxin in this group, shares 55 and 57 percent deduced amino acid sequence homology with SLT-I A and B subunits, respectively (Jackson *et al.*, 1987). Although SLT-II also uses Gb_3 as a functional cellular receptor (Wadell *et al.*, 1988), the Vero cell cytotoxicity of SLT-II cannot be neutralized by polyclonal or monoclonal anti-SLT-I antibodies (Jackson *et al.*, 1987). Another member of the SLT-II family is SLT-IIv (for SLT-II variant) which has 93 and 84 percent deduced amino acid sequence homology to the SLT-II A and B subunits, respectively (Weinstein *et al.*, 1988b). SLT-IIv is produced by *E. coli* strains that cause edema disease of swine (Weinstein *et al.*, 1988b; Marques *et al.*, 1987). SLT-IIv cytotoxicity is neutralized by polyclonal anti-SLT-II antibody. However, unlike SLT-II, SLT-IIv binds more avidly to globotetraosylceramide (Gb_4, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) than to Gb_3 (DeGrandis *et al.*, 1989; Samuel *et al.*, 1990). SLT-IIv was originally classified as a variant because it exhibits a 10,000-fold lower level of cytotoxicity on HeLa cells than Vero cells, whereas SLT-II is only 1- to 10-fold less cytotoxic for HeLa than Vero cells (Marques *et al.*, 1987). The differential

cytotoxicity of SLT-IIv appears to be due to the receptor specificity of this toxin and the concentration of Gb_4 on the sensitive and insensitive cells (Samuel *et al.*, 1990).

Recently, several new SLTs have been described that are considered members of the SLT-II group based on sequence homology or immunological cross-reactivity with SLT-II. These new SLT-II group members include: 1) SLT-IIva which is closely related to SLT-IIv by sequence homology (Gannon *et al.*, 1990); and 2) SLT-IIvha, SLT-IIvhb, and SLT-IIc which are nearly 97 percent homologous to SLT-II at the deduced amino acid sequence level (Ito *et al.*, 1990; Schmitt *et al.*, 1991).

The nomenclature that is currently used to identify the various SLT-II-related toxins is quite confusing. As mentioned, SLT-IIv was originally considered a "variant" based upon the 10,000 fold-difference in cytotoxicity observed on HeLa versus Vero cells. In 1990, Ito *et al.* identified two toxins produced from the human EHEC isolate B2F1 that were 97% homologous to SLT-II at the deduced amino acid level but were 100-fold less cytotoxic on HeLa versus Vero cells. These two toxins were therefore named SLT-IIvha and SLT-IIvhb for "SLT-II variant human" to differentiate them from the variant toxin produced by isolates that cause edema disease (SLT-IIv). In 1991, the operon that encodes SLT-IIc was isolated from strain E32511 and sequenced (Schmitt *et al.*, 1991). SLT-IIc was found to be highly homologous to SLT-II with only partial

serological cross-reactivity to SLT-II and a slight difference in cytotoxicity levels on HeLa versus Vero cells. At this time it was decided that SLT-IIvha and SLT-IIvhb, like SLT-IIc, were not "true variants" when compared to SLT-IIv. SLT-IIc was therefore assigned a lower case "c" to follow the SLT-IIvha and SLT-IIvhb nomenclature but without the addition of a "v". Since 1991, numerous other SLT-II-related toxins have been identified (Yamasaki *et al.*, 1991; Hii *et al.*, 1991; Tyler *et al.*, 1991; Paton *et al.*, 1992). Many of these toxins were identified by PCR analysis with specific primers, but only a few have been isolated and sequenced. In 1991, the nomenclature of *E. coli* cytotoxins was discussed at the WHO conference entitled "Shiga-like Toxin Producing *Escherichia coli* with a Special Emphasis on Zoonotic Aspects". During this meeting, it was recommended that all SLT-II-related toxins (including SLT-IIvha, SLT-IIvhb, and SLT-IIc) that appear to be closely related to SLT-II by DNA hybridization and sequence homology and exhibit only minor variations in other properties be designated "SLT-IIc". The lower case "c" was used in part to avoid confusion between the A and B subunits of the toxin and the toxin designation. For the purposes of this dissertation, the SLT-IIvha and SLT-IIvhb designations will be retained; however, newly described toxins (those isolated from strain H414-36/89) will be referred to as SLT-IIc. Collectively, SLT-IIvha, SLT-IIvhb,

and the newly identified SLT-IIc toxins will be called SLT-II-related toxins.

The specific sequence relationship among the SLT-II-related toxins is as follows. The processed A subunit of SLT-IIvha and SLT-IIvhb contains four amino acid differences from SLT-II, whereas the SLT-IIc A subunit is identical to SLT-II at the protein level (Fig. 1). In addition, the amino acid sequence of the processed B subunits of SLT-IIvha, SLT-IIvhb, and SLT-IIc are identical to one another and differ from the prototype SLT-II toxin by two amino acid residues (Ito *et al.*, 1990; Schmitt *et al.*, 1991) (Fig. 1).

Structure-function analysis of the A and B subunits.

A) A subunit. Site directed mutagenesis studies on *stx/slt*-I, *slt*-II, and the plant toxin ricin have identified the highly conserved glutamic acid residue at position 167 (Glu-167) in the A subunit of the SLT toxins and the equivalent Glu-177 in the ricin toxin A subunit to be important for the N-glycosidase activity of these toxins (Hovde *et al.*, 1988; Jackson *et al.*, 1990a; Schlossman *et al.*, 1989). These structure-function studies suggest that this residue lies within the catalytic site of the toxins. More recently, Gordon *et al.* (1992) demonstrated that a mutation of Glu-167 to glutamine in SLT-IIv reduced the cytotoxicity and enzymatic activity of SLT-IIv 10^6-fold and 1,500-fold, respectively. Moreover, weanling piglets that were vaccinated

Figure 1. Amino acid sequence comparison of SLT-II and the SLT-II-related toxins sequenced to date. SLT-II sequence taken from Jackson *et al.*, 1987; SLT-IIvha and SLT-IIvhb sequence taken from Ito *et al.*, 1990; SLT-IIc sequence taken from Schmitt *et al.*, 1991. (/) indicates signal sequence processing site for mature subunit protein. (*) indicates location of B-subunit mutagenesis (*slt*-IIvhb mutagenesis done by Dr. J. Samuel, and *slt*-IIc mutagenesis done by Dr. C. Schmitt).

A subunit

```
SLT-II     MKCILFKWVLCLLLGFSSVSYS/REFTIDFSTQQSYVSSLNSIRTEISTPLEHISQ
SLT-IIvha ----------------------/------------------T---------------
SLT-IIvhb ----------------------/--------------------------------
SLT-IIc   ----------------------/--------------------------------

SLT-II     GTTSVSVINHTH+GSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFY
SLT-IIvha -----------PP-----------------------------------------
SLT-IIvhb -----------PP-----------------------------------------
SLT-IIc   ----------------------------------------------------

SLT-II     YRFSDFTHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEF
SLT-IIvha ----------------------------------------------------
SLT-IIvhb ----------------------------------------------------
SLT-IIc   ----------------------------------------------------

SLT-II     SGNTMTRDASRAVLRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLN
SLT-IIvha ----------------------------------------------------
SLT-IIvhb ----------------------------------------------------
SLT-IIc   ----------------------------------------------------

SLT-II     WGRISNVLPEYRGEDGVRVGRISFNNISAILGTVAVILNCHHQGARSVRAVNEESQ
SLT-IIvha ----------------------------------------------------
SLT-IIvhb ----------------------------------------------M-----
SLT-IIc   ----------------------------------------------------

SLT-II     PECQITGDRPVIKINNTLWESNTAAAFLNRKSQFLYTTGK
SLT-IIvha ---------------------------------S-----E
SLT-IIvhb ---------------------------------S-----E
SLT-IIc   ----------------------------------------
```

B subunit

```
                                                          *       *
SLT-II     MKKMFMAVLFALASVNAMA/ADCAKGKIEFSKYNEDDTFTVKVDGKEYWTSRWNL
SLT-IIvha ------------V------/---------------N-------A-----------
SLT-IIvhb ------------V-----P/---------------N-------A-----------
SLT-IIc   ------------V------/---------------N-------A-----------


SLT-II     QPLLQSAQLTGMTVTIKSSTCESGSGFAEVQFNND
SLT-IIvha ----------------------------------
SLT-IIvhb ----------------------------------
SLT-IIc   ----------------------------------
```

with purified-mutant SLT-IIv did not exhibit any lesions typical of edema disease but developed a neutralizing titer to the wild-type SLT-IIv toxin. Taken together, these results further implicate Glu-167 as critical for the enzymatic activity of the SLTs.

B) B subunit. Jackson *et al.* (1990b) demonstrated that site-directed mutagenesis of the aspartate residues at positions 16 and 17 in the Shiga toxin B subunit resulted in a significant reduction in cytotoxicity but did not affect holotoxin assembly or immunoreactivity. These results suggest that the aspartate residues at positions 16 and 17 of the Shiga toxin B subunit may be part of the receptor binding site. Moreover, Stein *et al.* (1992) recently published X-ray crystallographic data which indicate that the Shiga toxin/SLT-I B subunit aspartate residue 17 is exposed in the proposed toxin-receptor binding region. These X-ray data provide more direct evidence that aspartate residue 17 is involved in carbohydrate binding. Studies by Samuel *et al.* (1990) on receptor-binding of SLT-II variant toxins demonstrated that SLT-IIvhb can bind to Gb_3 receptor analogs but with less affinity than SLT-II. Subsequent to these studies, Dr. J. Samuel used site-directed mutagenesis to assess whether the amino acid differences between SLT-IIvhb and SLT-II in the processed B subunit (see Fig. 1) would affect SLT-IIvhb toxin-receptor specificity. The asparagine codon at position 16 in the SLT-IIvhb B subunit

was mutagenized to an aspartate codon, and the alanine codon at position 24 in the SLT-IIvhb B subunit was also mutagenized to an aspartate codon. *E. coli* DH5α transformed with plasmids containing the mutated *slt*-IIvhb genes were used in this project.

Regulation of Shiga-like toxins. Expression of many known bacterial virulence factors is regulated by specific environmental conditions (Mekalanos 1992). SLT regulation studies indicate that Shiga-toxin/SLT-I production is regulated by iron (Calderwood *et al.*, 1987; Weinstein *et al.*, 1988a). An iron-Fur complex binds to the *stx/slt*-I promoter and inhibits transcription of the toxin operon (Calderwood *et al.*, 1987). Weinstein *et al.* (1988b) found that unlike SLT-I, SLT-II production is not iron regulated. Subsequent analysis of the *slt*-II toxin promoter by Sung *et al.* (1990) revealed that the *slt*-II promoter differs significantly from the *stx/slt*-I promoter and does not contain sequences where the Fur-iron repressor complex could bind. Another environmental signal that regulates virulence gene expression is temperature. Weinstein *et al.* (1988a) showed that Shiga toxin in an *S. dysenteriae* background is regulated by temperature but cloned *slt*-I in *E. coli* K-12 is not.

Role of Shiga toxin and the Shiga-like toxins in disease. There are no definitive data that prove a role for Shiga toxin

or SLTs in the clinical manifestations of hemorrhagic colitis or HUS because humans cannot be used as a model to study infections with *S. dysenteriae* or EHEC. There are, however, several indications that these toxins are centrally involved in disease. First, *Shigella dysenteriae* type 1 is the only *Shigella* spp. that produces Shiga toxin and is also the only *Shigella* spp. that has been associated with HUS (Prado *et al.*, 1986). Second, a human volunteer study by Levine *et al.* (1973) demonstrated that an *S. dysenteriae* chlorate-resistant mutant which lacked the structural gene for Shiga toxin (Neill *et al.*, 1988) produced a less severe febrile dysentery than that caused by the fully virulent parent strain. Third, a more recent investigation on *S. dysenteriae* pathogenesis demonstrated that a Shiga toxin deletion mutant of *S. dysenteriae* had a significantly decreased capacity to produce intestinal disease when fed to primates (Fontaine *et al.*, 1988). The intestinal damage observed from the tox^+ parent strain was characterized by destruction of the capillaries that serve the colonic mucosa (Fontaine *et al.*, 1988). One hypothesis on the relationship between toxin-mediated capillary damage and disease is that destruction of the colonic microvasculature may increase the access of SLTs and other bacterial products [e.g. lipopolysaccharide (LPS)] to the blood stream and thereby increase the risk of post-diarrheal HUS (Tesh *et al.*, 1991a). Fourth, numerous epidemiological studies have correlated infection with EHEC

that produce high levels of SLT and the development of HUS (Karmali *et al.*, 1985; Ratnam *et al.*, 1985; Marques *et al.*, 1986; Lopez *et al.*, 1989). Some of these epidemiological studies have incriminated the SLT-II group more than the Shiga toxin/SLT-I group in the development of microangiopathic sequelae. Specifically, epidemiological data from both Seattle (Ostraff *et al.*, 1989) and England (Scotland *et al.*, 1987) indicate that strains that produce SLT-II alone are more likely to cause hemorrhagic colitis that results in HUS or thrombotic thrombocytopenic purpura than those strains that produce SLT-I and SLT-II or SLT-I alone. Furthermore, EHEC animal model studies by Wadolkowski *et al.* (1990a) and Barrett *et al.* (1989) have shown a similar correlation between SLT-II production and kidney damage in mice and rabbits, respectively.

IV. Development of animal models to study the pathogenesis of EHEC infections.

Below is a brief description of several animal models developed to study EHEC pathogenesis. Although all of these models have contributed to the understanding of EHEC pathogenesis, none of the animals used in these models develop bloody diarrhea or damage to the renal glomeruli, both of which are hallmarks of human hemorrhagic colitis and HUS.

Infant rabbit model. Following two United States outbreaks of EHEC in 1982 (Riley *et al.*, 1983), an animal model was sought to help define the pathogenesis of the infecting agent, *E. coli* 0157:H7. Farmer *et al.* (1983) reported that five- to ten-day-old New Zealand white rabbits when intragastrically infected with a large dose of *E. coli* 0157:H7 develop watery diarrhea. Shortly thereafter, Potter *et al.* (1985) confirmed this finding. Although neither kidney damage nor bloody diarrhea was observed, this model was useful for studying the colonic histopathological lesions and the role of toxin in disease. Pai and co-workers (1986) further refined the rabbit model and used three-day-old intragastrically-inoculated rabbits. They described histological changes due to EHEC infection in the mid- and distal colon concomitant with the development of watery diarrhea in the rabbits. The histological changes included apoptosis in the surface epithelium, increased mitotic activity in the crypts, mucin depletion, and an infiltration of neutrophils in the lamina propria and epithelium (Pai *et al.*, 1986). Pai and co-workers further demonstrated that identical clinical and histopathological changes occur in three-day-old rabbits when purified SLT in a sodium bicarbonate buffer is administered intragastrically (Pai *et al.*, 1986). The latter finding supports a role for SLT in EHEC-mediated diarrhea. Two other studies support the concept that toxin alone may cause histopathological changes in the colon and development of

diarrhea in rabbits. In 1956, Cavanagh *et al.* reported that rabbits parenterally injected with 20 LD_{50} of purified Shiga toxin (thought to be free of endotoxin) from *Shigella shigae* (*S. dysenteriae*) develop watery diarrhea, cecal edema, and neurotoxemia. More recently, Barrett *et al.* (1989) found that continuous infusion of SLT-II into rabbits causes watery diarrhea and focal necrosis of the renal proximal convoluted tubules. Colonic lesions similar to those observed in humans with hemorrhagic colitis also occur.

Gnotobiotic piglet model. As mentioned previously, both Moon *et al.* (1983) and Tzipori *et al.* (1985) observed that gnotobiotic piglets infected with EPEC develop distinctive intestinal attaching and effacing (att-eff) lesions. More recently, two groups of investigators found similar att-eff lesions when 0157:H7 EHEC strains were fed to gnotobiotic piglets (Francis *et al.*, 1986; Tzipori *et al.*, 1986). Both Francis *et al.* and Tzipori *et al.* observed that EHEC colonize the piglet colonic epithelial cells, cause effacement of the microvilli, and pedestal formation of the cellular membrane that underlies the intimately attached bacteria. Watery diarrhea appears to be a direct consequence of the histopathological lesions and is dose dependent. Unlike the conclusions drawn from the rabbit model data (Pai *et al.*, 1986), Tzipori *et al.* (1987, 1989) found that when an SLT(-), plasmid-cured EHEC strain is fed to gnotobiotic piglets, the

colonic epithelial cell att-eff lesions and watery diarrhea are as severe as those caused by the parent strain. Tzipori *et al.* suggested, therefore, that toxin does not play a role in the observed colonic pathologies. The finding that fluid accumulation does not occur in SLT-injected ligated pig ileal loops (Tzipori *et al.*, 1987), however, suggests that gnotobiotic piglets may not be a relevant model for examination of toxin-associated colonic damage. Francis *et al.* (1989) found that oral inoculation of *E. coli* that produce SLT-II causes signs of central nervous system disease and histopathological damages in the brain of gnotobiotic piglets, whereas oral inoculation of non-SLT-II producing *E. coli* strains does not. Taken together, these results suggest that toxin produced by EHEC does not cause the att-eff damage to the pig colonic mucosa but may cause endothelial damage in the cerebellum and elsewhere.

Gnotobiotic calf model. Clinical and epidemiological data suggest that cattle may harbor EHEC (Montenegro *et al.*, 1990; Wells *et al.*, 1991). Natural EHEC infection in cattle has been shown to cause colonic att-eff lesions and watery diarrhea (Moxely *et al.*, 1986; Mainil *et al.*, 1987). Gnotobiotic calves experimentally infected with EHEC develop similar pathology in a dose-dependent manner (Moxely *et al.*, 1986).

Mouse models. Mouse models have also been used to study EHEC pathogenesis. Beery *et al.* (1984) demonstrated that culture filtrates from an *E. coli* 0157:H7 strain cause colonic necrosis, renal tubular damage, and hind-leg paralysis when parenterally injected in ICR mice. In 1987, Padhye *et al.* described a similar phenomenon upon injection of purified SLT. A more recent study by Xin-He *et al.* (1991), described the effects of a subcutaneous injection of 1 x 10^8 *E. coli* 0157:H7 into specific-pathogen-free mice. The mice appeared anorectic, lethargic, and inactive within 24 h of inoculation. Because the entire organism was injected parenterally, these findings probably reflect systemic growth of the organism and endotoxemia. Wadolkowski *et al.* (1990a) developed a mouse model to study the colonization capacity of 0157:H7 strains. She found that streptomycin-treated mice orally-infected with 0157:H7 strain 933cu-rev [a spontaneously derived mutant of strain 933 that had previously been cured of the large plasmid (Wadolkowski *et al.*, 1990a)] were colonized with the bacterium and died 5-13 d post-infection. Unlike the piglet and calf models, no intestinal histopathological damage was observed. However, acute bilateral tubular necrosis was apparent in the kidneys of orally-inoculated, moribund mice (Wadolkowski *et al.*, 1990a). Subsequent studies revealed that SLT-II not SLT-I production by the infecting organism is critical for the renal damage and death of the streptomycin-treated, orally-infected mice (Wadolkowski *et al.*, 1990b). One of the goals

of this dissertation project was to determine the virulence of several clinical EHEC isolates in this mouse model.

V. Description of the EHEC strains examined in this study.

The current EHEC screening techniques used by diagnostic laboratories in the United States focus on the prototypic 0157:H7 serotype. By contrast, some diagnostic laboratories in Canada, Great Britain, and Western Europe use alternative mechanisms for EHEC screening. These latter laboratories have isolated an abundance of non-0157:H7 *E. coli* associated with hemorrhagic colitis and HUS (Willshaw *et al.*, 1992; Bockemuhl *et al.*, 1992). One non-0157:H7 EHEC, 091:H21 strain B2F1, was isolated from a Canadian patient with HUS. This particular infection was traced to contaminated moose meat that the patient and his family had eaten [Dr. M. Karmali, (Hospital for Sick Children, Toronto, Ontario) personal communication]. Our laboratory obtained strain B2F1 from Dr. M. Karmali and found that it produced an SLT-II variant as indicated by cytotoxicity and toxin neutralization results (L. Marques and A. D. O'Brien unpublished results). The toxin elaborated by B2F1 was therefore called SLT-IIvh (variant human) to differentiate it from the variant SLT-II toxin isolated from weanling piglets with edema disease (SLT-IIv). While in our laboratory, Dr. J. Samuel made a cosmid bank of strain B2F1 and identified what appeared to be two copies of a gene that hybridized with an *slt*-IIv probe. Shortly thereafter, Ito

et al. (1990) published the sequence of two *slt*-II-related toxin genes cloned from this strain: *slt*-IIvha and *slt*-IIvhb. Since that time, several other clinical isolates have been identified that have more than one copy of *slt*-II (Schmitt *et al.*, 1991; Hii *et al.*, 1991; and Tyler *et al.*, 1991). Schmitt *et al.* (1991) used *in situ* gel hybridization to identify two such strains: 0157:H- strain E32511 and 0157:H7 strain J-2. Both E32511 and J-2 contain one copy of *slt*-II and one copy of *slt*-IIc (Schmitt, *et al.*, 1991; Yamasaki *et al.*, 1991). In addition, a derivative of strain E32511 which lost the SLT-II toxin-converting phage contains only the *slt*-IIc toxin gene (Hii *et al.*, 1991). This derivative strain is designated E32511/HSC. During the course of my studies, we obtained 091:H21 strain H414-36/89 from Dr. S. Aleksic (Hygienisches Institut, Hamburg) in Germany. Strain H414-36/89 was isolated from a patient with hemorrhagic colitis and was reported to produce SLT-II as determined by DNA colony-blot and toxin-neutralization results (Bockemuhl *et al.*, 1992). In this dissertation project, the virulence of isolates B2F1, H414-36/89, E32511, E32511/HSC, and J-2 was assessed in the orally-infected mouse model. In addition, the toxins produced by strain B2F1 and H414-36/89 were cloned and characterized.

VI. Specific Aims

The major objective of my research project was to characterize O91:H21 EHEC isolates B2F1 and H414-36/89. The three specific aims designed to achieve this objective were as follows. First, the virulence of the O91:H21 strains was compared to several SLT-II-producing EHEC isolates in an orally-infected, streptomycin-treated mouse model. Second, the basis for the enhanced virulence of 091:H21 strain B2F1 and 091:H21 strain H414-36/89 was analyzed by several parameters. The role of SLT-II-related toxin production in pathogenicity of orally-infected, streptomycin-treated mice was determined by passive immunization of the mice with anti-SLT-II monoclonal antibody prior to oral infection with either strain B2F1 or strain H414-36/89. In addition, the capacity of strain B2F1 to co-colonize orally-infected mice with other less virulent EHEC strains was evaluated. Finally, the location of intestinal colonization of strain B2F1 or strain H414-36/89 was examined by obtaining viable bacterial counts from isolated intestinal epithelial cells or whole intestinal segments from B2F1- or H414-36/89-infected mice.

The third aim of this project was to identify and characterize the toxin operons from the two mouse-virulent 091:H21 strains. The number of toxin operons present in strain B2F1 and H414-36/89 was assessed by *in situ* gel hybridization, and a total of four toxin operons were cloned. Phage induction experiments were done to determine whether the

slt operons, like *slt*-I and *slt*-II in other isolates, were located on toxin-converting phage. A cytotoxicity and immunoreactivity profile of the cloned SLT-II-related toxins was obtained and attempts to isolate an SLT-IIvhb-specific monoclonal antibody were made. The toxicity of the SLT-II-related toxins isolated from strain B2F1 and the prototypic SLT-II toxin were compared *in vivo* and *in vitro*. The *in vivo* studies were accomplished in two steps: 1) DH5α(Str^r) strains transformed with toxin-producing clones were administered orally to streptomycin-treated CD-1 mice; 2) SLT-IIvhb and SLT-II were purified, and a comparison was made of the specific activities of these two toxins intraperitoneally-injected into CD-1 mice. The regulation of toxin gene expression by environmental growth temperatures was analyzed with an *slt*-IIvhb-*pho*A reporter gene fusion. As a final step in the characterization of the SLT-II-related toxins, B-subunit mutants of SLT-IIvhb which had previously been constructed to mimic the amino acid sequence of the SLT-II B-subunit were analyzed to determine the amino acids critical for the SLT-II-related toxin phenotype.

Materials and Methods

Plasmids and bacterial strains. Bacterial strains and plasmids used in this dissertation are described in Tables 2 and 3, respectively. For the *in vivo* studies, a spontaneous streptomycin-resistant (Str^r) mutant was obtained from each of the clinical EHEC isolates and K-12 strain DH5α.

Media, enzymes, and biochemicals. Bacteria were routinely cultured in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982). Clinical EHEC strains were plated on MacConkey agar (Difco, Detroit, Mich.). Where indicated or required for selective pressure, the growth medium was supplemented with antibiotics (Sigma Chemical Co., St. Louis, Mo.) at the following concentrations: streptomycin, 30 μg/ml; nalidixic acid, 50 μg/ml; rifampin, 50 μg/ml; ampicillin, 200 μg/ml for pBluescript vectors (Stratagene, La Jolla, Calif.), 100μg/ml for pBR329 and pHC79. Restriction enzymes, RNase, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Fetal calf serum (silver grade) was obtained from ICN Biomedical Inc. (Irvine, Calif.), and Eagles Minimum Essential Medium was purchased from Whittaker Bioproducts, Inc. (Walkersville, Md.)

Plasmid analysis of the EHEC isolates. Plasmid DNA was isolated from strains B2F1, H414-36/89, E32511, E32511/HSC,

Footnotes for Table 2

[a] The designation IIc includes all unnamed SLT-II-related toxins.

[b] NA, Not Applicable.

Table 2. *E. coli* strains used in this study

Strain	Serotype	SLT toxin gene(s)	Reference
EHEC strains			
B2F1	091:H21	IIvha, IIvhb	Ito *et al.*, 1990
H414-36/89	091:H21	II, IIc[a], IIc	This study
J-2	0157:H7	II, IIc	Yamasaki *et al.*, 1991
933	0157:H7	I, II	Strockbine *et al.*, 1986
E32511	0157:H-	II, IIc	Schmitt *et al.*, 1991
E32511/HSC	0157:H-	IIc	Hii *et al.*, 1991
Other E. coli			
C600	rough	NA[b]	Silhavy *et al.*, 1984
DH5α	rough	NA	ibid.

Table 3. Recombinant plasmids used in this study

Plasmid	Toxin	Description	Reference or source
pJES120	SLT-II	2.4 kb *Bam*HI-*Eco*RI fragment of *slt*-II from pLMS2.2 (Sung et al., 1990) in pSK(-)[a]	Dr. J. Samuel
pLP32	SLT-II	*slt*-II in pBS[a]	Schmitt *et al.*, 1991
pMJ100	SLT-II	*slt*-II in pBS[a]	Weinstein *et al.*, 1989
pSQ12	SLT-IIvha	*slt*-IIvha from B2F1 in pHC79	This study
pSQ343	SLT-IIvha	2.3 kb *Pst*I-*Eco*RV fragment from pSQ12 in pKS(-)[a]	This study
pSQ347	SLT-IIvha	2.3 kb *Bam*HI-*Hind*III fragment from pSQ343 in pACYC184	This study
pJES54	SLT-IIvhb	*slt*-IIvhb from B2F1 in pHC79	Dr. J. Samuel
pJES210	SLT-IIvhb	*slt*-IIvhb from B2F1 in pHC79	Samuel *et al.*, 1990

Plasmid	Toxin	Description	Reference or source
pSQ541	SLT-IIvhb	4.0 kb *Sal*I-*Eco*RI fragment from pJES54 in pHC79	This study
pSQ543	SLT-IIvhb	2.8 kb *Pst*I-*Eco*RI fragment from pJES54 in pSK(-)[a]	This study
pSQ544	SLT-IIvhb	4.0 kb *Sal*I-*Eco*RI fragment from pSQ541 in pSK(-)[a]	This study
pSQ544-2	SLT-IIvhb	4.0 kb *Sal*I-*Eco*RI fragment from pSQ541 in pKS(-)[a]	This study
pSQ545	SLT-IIvhb	2.8 kb *Pst*I-*Eco*RI fragment from pSQ543 in pKS(-)[a]	This study
pSQ547	SLT-IIvhb	2.8 kb *Bam*HI-*Hind*III fragment from pSQ543 in pACYC184	This study
pSQ549	SLT-IIvhb	2.8 kb *Bam*HI-*Hind*III fragment from pSQ543 in pBR328	This study

Plasmid	Toxin	Description	Reference or source
pSQ47	SLT-IIc	*slt*-IIc from H414-36/89 in pSK(-)[a]	This study
pSQ81	SLT-II	*slt*-II from H414-36/89 in pSK(-)[a]	This study
pSQ135	SLT-IIc	*slt*-IIc from H414-36/89 in pSK(-)[a]	This study
pSS1324	N/A	source of *pho*A gene for gene fusion	Stibitz *et al.*, 1991
pSS1295	N/A	vector pTTQ8 with unique *Xho*I site	ibid.
pSQ448	N/A	*slt*-IIvhb A subunit-*pho*A gene fusion cloned into *Bam*HI-*Hind*III of pSS1295	This study
pRK415	N/A	Low copy cloning vector	Keen *et al.*, 1987
pSQ19	N/A	1.9 kb *Sal*I-*Eco*RV fragment from pSQ544 in pKS(-)[a]	This study

[a] Plasmids purchased from Stratagene: pBS, pBluescript; pSK, pBluescript SK; pKS, pBluescript KS.

933, and an isogenic plasmid-cured derivative of 933 (933cu). The isolation procedure was that described in the Qiagen kit (Qiagen Inc., Chatsworth, Calif.). The isolated DNA was subjected to agarose gel (0.4%) electrophoresis for 15 h at 20 V followed by 5 h at 50 V. Gels were stained with ethidium bromide.

To determine whether any large plasmids observed in the EHEC plasmid profiles were related to the O157 EHEC plasmid, samples of EHEC plasmid DNAs were digested with *Hind*III, separated by agarose gel electrophoresis (0.9%), and subjected to *in situ* gel hybridization as described by Kidd *et al.* (1983) with the following modifications. After electrophoresis, the agarose gel was washed in 0.5 N NaOH/0.15 M NaCl to denture the DNA. The gel was then washed in a neutralization solution (0.5 M Tris-HCl pH 8.0/ 0.15 M NaCl) and dried under vacuum at 60°C with a Bio-Rad Laboratories (Richmond, Calif.) slab gel dryer. The resultant gel was placed in a heat sealed bag with hybridization solution [150mM NaCl, 15mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS, ^{32}P-labelled DNA probe (1.0-3.0 x 10^8 cpm/μg)] which also contained 20 mM sodium pyrophosphate as a blocking agent. The probe used for this experiment was a 3.4 kb *Hind*III fragment originally isolated from EHEC strain 933 plasmid DNA (Levine *et al.*, 1987); the probe was labeled with [^{32}P]dCTP (New England Nuclear Research Products, Boston, Mass.) by nick-translation (BRL Nick Translation System, Life Technologies,

Gaithersburg, Md.). The gel hybridization reaction mixture was incubated at 68°C overnight and then washed as follows: one wash in 2X SSC (0.30 M NaCl, 0.03 M sodium citrate, pH 7.0) at 68°C for 1 h and then one wash in 0.1 X SSC at 68°C for 1 h. The washed gel was then exposed to X-OMAT film (Eastman Kodak Company, Rochester, N.Y.).

Mouse feeding, colonization, and oral 50% lethal dose (LD_{50}) studies. Five- to six-week-old male CD-1 outbred mice were used throughout this project (Charles River Laboratories, Wilmington, Mass.). The methods for mouse feeding and colonization were as described by Myhal *et al.* (1982) with the following modifications. Throughout each experiment, mice were given drinking water containing streptomycin (5 g/l). Streptomycin treatment reduces the level of facultative anaerobic bacteria that normally colonize the mouse intestine (Myhal *et al.*, 1982). After 24 h of streptomycin treatment, the food from groups of 3 to 10 mice was removed for the night. The following day, saturated overnight cultures of (Str^r) *E. coli* were washed in phosphate-buffered saline (PBS) and then resuspended in 20% sucrose at a tenth of the original culture volume. Each mouse was fed approximately 1 x 10^{10} colony forming units (CFU) of *E. coli* in a 1 ml sucrose suspension. Within 1-2 h, the mice consumed the bacteria and were returned to their normal diet. Groups of 3 to 5 mice fed DH5α(Str^r) that contained toxin genes cloned into vectors with

ampicillin resistance markers were given drinking water with ampicillin (5 g/l) and streptomycin (5 g/l).

Bacterial colonization of orally-inoculated, individually-housed mice was assessed by persistence of the test organism in feces over time. Mouse cages were changed daily. On designated days, a total of 1 g of feces was isolated from each cage, suspended in 10% tryptone (Difco), and homogenized with a Stomacher 80 lab blender (Tekmar, Cincinnati, Ohio). Ten-fold dilutions of the fecal homogenate were made and plated on MacConkey agar that contained streptomycin. Colonization is expressed as *E. coli* CFU/g feces.

If all animals in a group fed 10^{10} CFU of an EHEC strain died, groups of five animals were fed graded doses of the organism to assess the oral LD_{50}. The initial 10^{10} CFU suspension was diluted in 20% sucrose to yield the dose required in a 50 μl sample. Each 50 μl aliquot was fed to mice using 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 (1938).

Histological studies. 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 node, 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 by Dr. Jenny Burris, a veterinary pathologist, for histopathologic changes.

Hematological analyses. Blood was obtained from the carotid artery of experimental mice. Whole blood was assessed for hemoglobin concentration, hematocrit, and platelet count with a Baker 9000 Cell Counter (Serono Baker Diagnostics, Allentown, Penn.). Serum was isolated from whole blood and tested for concentration of Blood Urea Nitrogen (BUN), creatinine, and total protein using the Baker Centrifichem 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 ascitic fluid which contained either IgG monoclonal anti-SLT-II B subunit antibody [designated BC5BB12 (Downes *et al.*, 1988)] or a control IgG monoclonal anti-SLT-I B subunit antibody [designated 13C4 (Strockbine *et al.*, 1985)]. The injections were given to groups of 5 to 10 mice one day before and again immediately

prior to oral inoculation with *E. coli* 091: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 streptomycin-treated mouse intestine was evaluated as follows. Groups of four streptomycin-treated mice were fed 10^{10} CFU of each *E. coli* strain to be tested. The animals were sacrificed when moribund or 18-24 h prior to the estimated time of death based on preliminary studies. Intestinal segments of 3 cm in length 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 PBS through the bowel lumen with a sterile 22 gauge needle attached to a 1 cc 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 (Weiser *et al.*, 1973). No attempts were made to remove bacteria from the epithelial cells prior to plating. The homogenized intestinal segments and the epithelial cell suspensions were diluted in PBS and plated in duplicate on MacConkey agar containing streptomycin to determine CFU per intestinal segment.

DNA hybridization studies to determine toxin gene copy number. Total DNA was isolated from various EHEC isolates as described

by Wilson (1989), digested with either *Eco*RI or *Hind*III, and the fragments separated by agarose gel (0.9%) electrophoresis. The agarose gels were then probed by *in situ* gel hybridization and washed under stringent conditions as previously detailed. The *slt*-II probe used was a 1 kb *Sma*I-*Sca*I *slt*-IIvha 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 3) and was constructed to identify all known members of the *slt*-II toxin group. The *slt*-II DNA probe was labeled with [^{32}P]dCTP (New England Nuclear Research Products) by nick translation (BRL Nick Translation System).

Recombinant DNA techniques. Plasmid DNA was purified by alkaline lysis (Birnboim *et al.*, 1979) or by Qiagen preparation (Qiagen Inc. Chatsworth, Calif.). DNA fragments used for subcloning were separated by 0.9% gel electrophoresis and eluted from the gel matrix with the Geneclean system (Bio101 La Jolla, Calif.). Ligated fragments were transformed into *E. coli* K-12 strain DH5α made competent for uptake of DNA by calcium chloride and heat shock (Mandel *et al.*, 1970).

Cloning the *slt*-IIvha toxin gene from 091:H21 strain B2F1. A cosmid bank of strain B2F1 was previously constructed by Dr. J. Samuel (Samuel *et al.*, 1990). Two cosmid clones which contain *slt*-IIvhb (pJES54 and pJES120) were used in this study

(Table 3). The second *slt* gene present in strain B2F1, *slt*-IIvha, was isolated as follows. The B2F1 cosmid bank was transformed into competent *E. coli* HB101 cells. Transformants were screened by DNA colony-blot hybridization for the presence of *slt* with an 800bp *Sma*I-*Pst*I *slt*-II DNA probe as described by Sambrook *et al.* (1989). Plasmid DNA from putative toxin clones was isolated, digested with *Pst*I, and separated by 0.9% agarose gel electrophoresis. The agarose gels were probed with the *slt*-II probe by *in situ* gel hybridization and analyzed for the presence of a 4.3 kb *Pst*I fragment previously reported to contain the *slt*-IIvha gene (Ito *et al.*, 1990).

Cloning toxin genes from EHEC strain H414-36/89. *In situ* gel hybridization of *Eco*RI digested H414-36/89 DNA identified fragments of 4.1 kb, 8.4 kb, and 12.5 kb in length that contained an *slt*-II or *slt*-II-related toxin gene. As a first step toward isolating the toxin genes from these fragments, total H414-36/89 DNA was digested with *Eco*RI and separated by agarose gel (0.7%) electrophoresis. Regions of the agarose gel corresponding to DNA of 4-5 kb, 8-9 kb, and 12-14 kb in length were isolated. DNA was purified from each of the gel slices by the Geneclean system (Bio101, La Jolla, Calif.) and then amplified by the polymerase chain reaction (PCR) with either Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) or Vent polymerase (New England Biolab, Beverly, Mass.) in a

thermocycler (Perkin-Elmer Cetus). Primers of 21 and 25 base pairs in length were used for the amplification (Fig. 2); these primers had previously been designed by Dr. C. Schmitt 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 a unique *Sau*3A and *Eco*RI site 5' and 3' to the toxin gene, respectively. The PCR products were isolated, purified by the Geneclean system (Bio101), digested with both *Sau*3A and *Eco*RI, and ligated into *Bam*HI-*Eco*RI-digested pBluescript vector pSK(-) (Stratagene). Ligated fragments were transformed into competent DH5α. Ten bacterial colonies from each transformation reaction were selected, grown overnight, and centrifuged. Plasmid DNA was isolated from the bacterial pellet of each culture by alkaline lysis (Birnboim *et al.*, 1979) and assessed for the presence of a toxin gene fragment in the recombinant plasmid by restriction map analysis. In addition, culture supernatants from each of the ten saturated bacterial cultures were assayed for Vero cell cytotoxicity.

Cytotoxicity and toxin 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 (1980)

```
SQPF1   5'  CAG CTC GAG TTT CAC CCG TTG TAT ATA AAG ACT GT  3'

SK          TCT AGA ACT AGT GGA TC

                      *
CKS1        TGA GAG CGA TCG ACT CAT AAT

                           *
CKS2        ACT GAC TGA ATT CTG ACA CAG ATT A
```

Figure 2. Sequence of primers used in this study. SQPF1 is a 35-mer used for the production of a unique *Xho*I site in place of the ochre codon for translational termination of the SLT-IIvhb A subunit. The sequence CTC GAG T in SQPF1 created the *Xho*I site. CKS1 and CKS2 were used for the isolation of the *slt*-II genes from O91:H21 strain H414-36/89. The "*" indicates a base-pair change from the native *slt*-II sequence which created unique restriction sites 5' and 3' of the PCR product.

as modified by Schmitt *et al.* (1991). The cytotoxic titer per milliliter culture is expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a given well (CD_{50}/ml). Cytotoxin immunoreactivity assays were done by incubating a single dilution of antibody with serial 2-fold dilutions of toxin for four hours at 37°C. The toxin-antibody mixtures were then added to Vero cells. The antibodies and dilutions used for cytotoxicity immunoreactivity experiments were as follows: polyclonal anti-SLT-II [designated AJ65 (Strockbine *et al.*, 1986)] at a 1:50 dilution, undiluted culture fluid of monoclonal anti-SLT-II A subunit [designated 2E1 (Perera *et al.*, 1988)], ascites of monoclonal anti-SLT-II B subunit [designated BC5BB12 (Downes *et al.*, 1988)] at a 1:100 dilution, and undiluted culture fluid of monoclonal anti-SLT-I A subunit [designated 13C4 (Strockbine *et al.*, 1985)]. The antibody dilutions were significantly more concentrated from what would normally be used for a standard SLT-II cytotoxin neutralization assay and were determined from preliminary neutralization experiments designed to neutralize the majority of SLT-II-related toxin antigen.

Bacteriophage induction, plaquing, and lysogen isolation. LB broth and agar supplemented with 10 mM $CaCl_2$ were used throughout all the phage experiments. Phage were induced from strain H414-36/89 by mitomycin C induction (Marques *et al.* 1987). Induction of bacteriophage from strain B2F1 was

attempted by three different methods: mitomycin C induction as described by Marques *et al.* (1987), ultraviolet induction as described by O'Brien *et al.* (1984), and spontaneous induction as described by H. W. Smith (personal communication, now deceased). The method for spontaneous induction of phage from strain B2F1 was as follows. A 0.4 ml sample of an overnight culture of *E. coli* strain B2F1 or control strain C600 was inoculated into 10 ml LB. The cultures were incubated at 37°C without shaking for 5-6 h. The culture supernatants were separated from the bacteria by centrifugation and filtered to remove bacterial debris. These supernatants (putative phage lysates) and supernatants from the ultraviolet and mitomycin C induction experiments were stored at 4°C with one drop of chloroform. The supernatants were then tested for infection-competent phage particles by plaquing samples on an *E. coli* K-12 host. A 0.1 ml aliquot of a putative phage lysate was incubated for 20 min at 37°C with an equal volume of an *E. coli* C600 culture that had reached an optical density at 600 nm of approximately 0.300. The mixture was then added to soft LB-agar and poured onto hard LB-agar plates. Lysogens of ϕH414 were isolated from colonies at the periphery of three purified plaques.

Southern blot analysis of phage DNA. Because infection-competent phage were not isolated from EHEC strain B2F1, Southern blot analysis was done on total B2F1 DNA to identify

any phage sequences present in this strain. The Southern blot was done as described by Maniatis *et al.* (1982). Total DNA previously isolated from B2F1, ϕH-19J (SLT-I-converting phage from 026:H11 EHEC strain H-19), and ϕ933W (SLT-II-converting phage from EHEC strain 933) was digested with *Eco*RI and run on a 0.9% agarose gel. The digested DNA was then transferred to nitrocellulose and hybridized with total DNA from ϕH-19J and ϕ933W that had been digested with *Eco*RI and labeled with [^{32}P]dCTP (New England Nuclear Research Products) by nick translation (BRL Nick Translation System). The Southern blot was washed under high stringency conditions (22% mismatch) before exposure to X-OMAT film (Eastman Kodak Company).

Monoclonal anti-SLT-IIvhb antibody production. The antigen used for the production of monoclonal antibodies was a crude SLT-IIvhb toxoid. The toxoid was prepared from a bacterial cell pellet of an overnight culture of *E. coli* HB101/pJES210 that was concentrated 10-fold in PBS and disrupted by sonication. The resultant sonic lysate was incubated with 1.0% formaldehyde at 37°C for 48 h after which the formaldehyde was removed from the preparation by dialysis against PBS. The formaldehyde-treated lysate was tested for Vero cell cytotoxicity and found to have some residual activity (10^2 CD_{50}/ml). However, a 100 μl dose of this toxoid was at or below the published mouse LD_{50} for SLT-II (Yutsudo *et al.*, 1987).

The immunization protocol was as follows. Four- to five-week-old female BALB/cJ mice (Jackson Laboratories, Bar Harbor, Maine) were inoculated intraperitoneally with 0.2 ml of a 1:1 (vol/vol) mixture of toxoid and complete Freund's adjuvant. Twenty-five days later, mice were boosted intraperitoneally with 0.2 ml of a 1:1 mixture of toxoid and incomplete Freund's adjuvant. After mice received two additional intraperitoneal boosts, the animals were given 0.2 ml of SLT-IIvhb toxoid in PBS (1:1) intravenously via the tail vein. Mice were sacrificed five days after the intravenous inoculation, and mouse splenocytes were fused to Sp2/0 cells (BALB/c myeloma cell line) with polyethylene glycol as described by Harlow *et al.* (1988). Hypoxanthine, aminopterin, and thymidine (HAT) (Whittaker Bioproducts) selection was used to isolate hybridoma clones. On day 30 post-fusion, culture supernatants from the resultant hybridomas were screened for SLT-IIvhb-neutralization. Crude SLT-IIvhb samples at doses of 20, 50, and 100 Vero CD_{50} were incubated with serial 2-fold dilutions of hybridoma supernatant for 4 h. The putative antibody-toxin mixtures were then added to semi-confluent monolayers of Vero cells and further incubated for 48 h at 37°C. Cloning of the hybridomas was unsuccessful. No clones with significant cytotoxin neutralizing activity were isolated.

Toxin purification. Both SLT-II and SLT-IIvhb were purified from an *E. coli* strain DH5α which contained the appropriate toxin gene on a high-copy number pBluescript plasmid (Stratagene). The purification method is based on a protocol developed by O'Brien *et al.* (1983a) for the purification of Shiga toxin/SLT-I from *E. coli*. Saturated overnight cultures (36 ml) of DH5α/pJES120 (SLT-II) or DH5α/pSQ543 (SLT-IIvhb) were inoculated into a total of 6 liters of enriched media (2% tryptone, 1% yeast extract, 0.5% NaCl, and 0.2% glycerol). The bacteria were cultured for 24 h, harvested by centrifugation, and lysed by sonic disruption. The bacterial lysates were clarified by centrifugation and concentrated with 60% ammonium sulfate. The resultant crude toxin preparations were dialyzed against 0.05M Tris pH 7.0 (SLT-II) or 0.05M Tris pH 8.0 (SLT-IIvhb), subjected to CL-6B DEAE-sepharose anion exchange chromatography (Pharmacia, Uppsala, Sweden) with the same buffer, and eluted with a 0-1.0 M NaCl gradient. The fractions that contained both the highest level of Vero cell cytotoxicity and the lowest level of protein concentration (determined by BCA reagent assay, Pierce, Rockford, Ill.) were pooled and dialyzed against chromatofocusing starting buffer (SLT-II: 0.025M histidine- HCl, pH 6.2; SLT-IIvhb: 0.025M imidazole-HCl, pH 7.4). The toxin preparations were then applied to a chromatofocusing column [Polybuffer-exchanger 94 (Pharmacia)] that had been equilibrated with the appropriate starting buffer. Fractions were eluted from the column with

Polybuffer 74-HCl pH 4.0 (Pharmacia). The toxin fractions with the highest cytotoxicity and lowest protein concentration were pooled and dialyzed against PBS. The toxin preparations were then subjected to immunoaffinity chromatography with monoclonal anti-SLT-II B subunit antibody [designated BC5BB12 (Downes *et al.*, 1988)] as the adsorbent. Eight 1 ml fractions were eluted from the immunoaffinity column with 0.1 M glycine pH 2.8 and immediately neutralized with 50 μl of 1 M Tris pH 9.0. The cytotoxic activity and the homogeneity of each fraction were assessed by Vero cell cytotoxicity and denaturing polyacrylamide gel electrophoresis, respectively. The fraction(s) containing the highly purified toxin protein were dialyzed against PBS and stored at 4°C. Purified toxin protein concentrations were determined in triplicate using a Micro-BCA protein assay reagent kit (Pierce).

Toxin LD_{50} studies. The quantity of purified toxin required to kill mice was assessed by 50% lethal dose (LD_{50}) studies. Various dilutions of purified SLT-II or SLT-IIvhb in 0.2 ml sterile non-pyrogenic 0.9% saline (Kendall McGaw Laboratories, Inc., Irvine, Calif.) were administered intraperitoneally to groups of five CD-1 mice. Deaths were monitored daily, and LD_{50} values were determined by the method of Reed and Meunch (1938).

Denaturing and non-denaturing polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)-PAGE of both purified and crude toxin protein preparations was done using the Mini-Protean II slab cell according to the instruction manual provided by the manufacturer (Bio-Rad). Discontinuous slab gels (Laemmli *et al.*, 1970) with a 12.0% acrylamide resolving gel and 4.0% acrylamide stacking gel were routinely used. Discontinuous non-denaturing gels were electrophoresed in the Mini-Protean II slab cell (Bio-Rad) with a Tris/glycine running buffer (0.2 M glycine brought to pH 8.9 with Tris base). A 12.5% acrylamide resolving gel and 4.0% acrylamide stacking gel were used. The non-denaturing polyacrylamide gel matrices were made in the Tris/glycine running buffer from a stock solution of 30:0.8 Acrylamide:Bis. Proteins were mixed 1:1 by volume with sample buffer [glycerol-2X Tris/glycine running buffer-water (2:2:1, by volume)] and immediately loaded onto the gel. The non-denaturing gels were run at 150 V constant voltage for 1.5 h. Both the denaturing and the non-denaturing gels were fixed in water-methanol-acetic acid (60:40:10, by volume) and stained with either Coomassie brilliant blue R-250 (Bio-Rad) or silver stain (Bio-Rad silver stain kit).

Western blot and immunodot blot analysis. Western blot analysis was used after either denaturing or non-denaturing PAGE to identify specific toxin proteins from each step of the

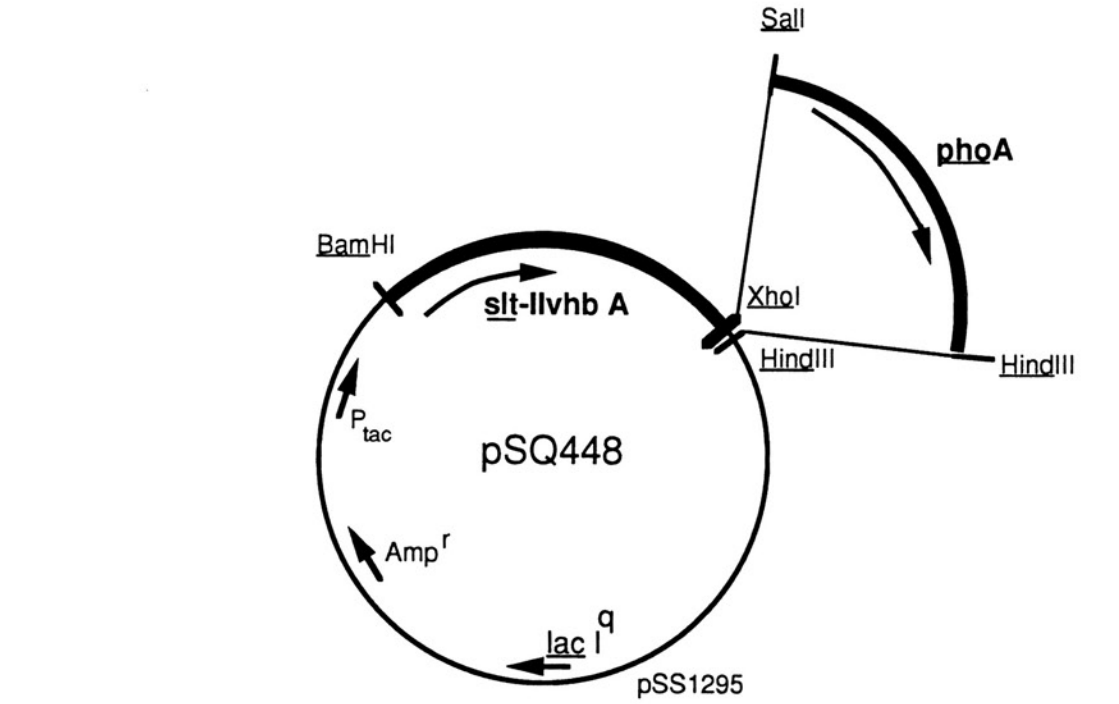
toxin purification procedure and also to identify the presence of an SLT-IIvhb-alkaline phosphatase protein fusion developed during this project. A comparative immunodot blot analysis was used to determine the amount of SLT protein present in various crude toxin preparations. Although the protein transfer method was different between the Western blot and the immunodot blot, detection of the transferred proteins was essentially the same for both procedures. For the Western blot, the electrophoretic transfer of proteins from a polyacrylamide matrix to polyester-supported BAS-NC™ nitrocellulose (Schleicher & Schuell, Inc. Keene, N.H.) was done in a Mini-trans-blot electrophoretic transfer cell according to the instruction manual provided by the manufacturer (Bio-Rad). For the immunoblot, serial dilutions of either sonically-disrupted bacterial cultures or purified toxin preparations were spotted onto BAS-NC™ nitrocellulose (Schleicher & Schuell) through a 96-well dot-blot apparatus (Schleicher & Schuell) connected to a vacuum. Analysis of the transferred proteins from both the Western blot and the dot-blot was done as follows. The nitrocellulose was air dried and incubated for 30 min at room temperature in PBS with 0.1% Tween 20 (Bio-Rad) (PBS-T) and 5% non-fat dried milk (Carnation Company, Los Angeles, Calif.). The Western blot was then incubated for 1 h with antibody diluted in PBS-T at the following concentrations: polyclonal anti-SLT-II [designated AJ65 (Strockbine *et al.*, 1986)] at a 1:100

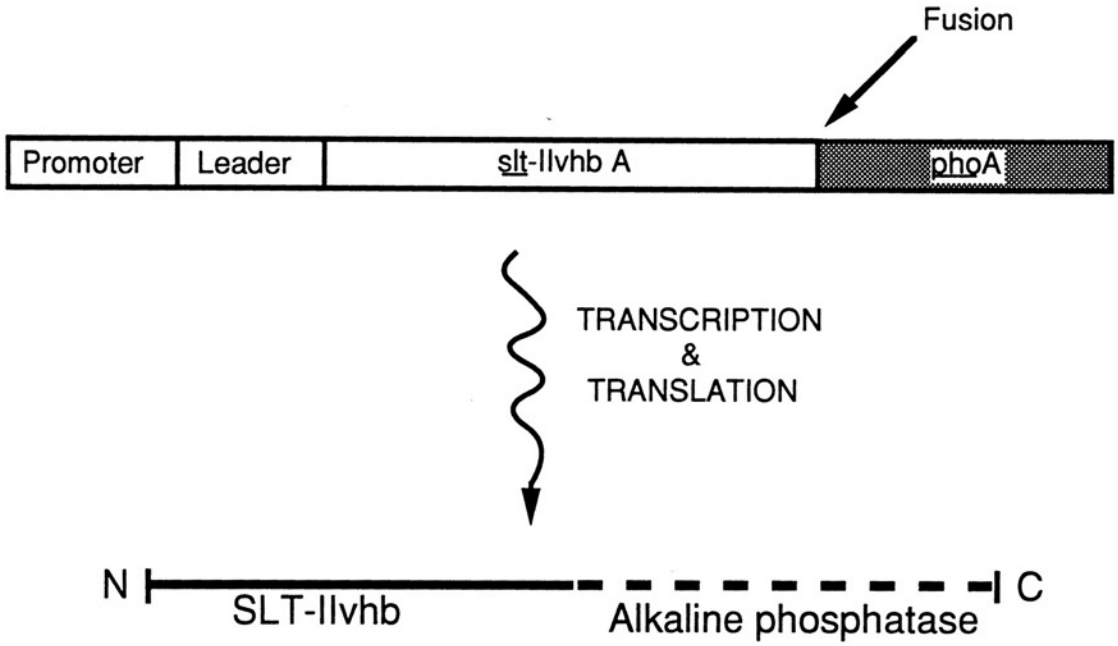
dilution, polyclonal anti-SLT-IIvh antibody [designated αSLT-IIvh (gift from Dr. Y. Takeda)(Oku *et al.*, 1989)] at a 1:500 dilution, polyclonal anti-alkaline phosphatase antibody at a 1:100 dilution (gift from Dr. S. Stibitz), monoclonal anti-SLT-II A [designated 11E10 (Perera *et al.*, 1988)] at a 1:5 dilution, or monoclonal anti-SLT-II B [designated BC5 (Downes *et al.*, 1988)] at a 1:500 dilution. The anti-SLT-II monoclonals do not react with toxins transferred from denaturing PAGE. The dot-blot membrane was washed with PBS-T and then incubated with a 1:5 dilution of anti-SLT-II A subunit monoclonal antibody [designated 11E10 (Perera *et al.*, 1988)] in PBS-T for 1 h. The membranes were washed three times for 5 min each in PBS-T at room temperature and then incubated with a 1:500 dilution of either horseradish peroxidase-conjugated goat anti-rabbit immunoglobin G (IgG) antibody (Bio-Rad) in PBS-T or horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad) in PBS-T for 1 h. Next, the membranes were again washed three times for 5 min each in PBS-T, and the antigen-enzyme-linked antibody reactions were visualized with either the Bio-Rad HRP color development reagent 4-chloro-1-napthol for Western blot analysis or the more sensitive ECL Western blotting detection reagent (Amersham International, Amersham, U.K.) for dot-blot analysis. The intensities of the dot-blot color reactions were estimated visually.

Construction of *slt*-IIvhb-*pho*A fusion and measurement of alkaline phosphatase activity. An SLT-IIvhb A subunit-alkaline phosphatase protein fusion was constructed under the direction of Dr. S. Stibitz for the SLT-IIvhb regulation studies (see Fig. 3). To construct the fusion, a unique *Xho*I site was created by PCR technology in *slt*-IIvhb in place of the ochre codon for A subunit translational termination. The primer SQPF1, a 35 bp oligomer which contained the *Xho*I site, and the SK primer (Stratagene) (Fig. 2), were used as amplimers for the *slt*-IIvhb A subunit gene from pSQ543 DNA. The PCR reaction product was directionally ligated into the *Bam*HI and *Xho*I sites of vector pSS1295 (Table 3). The *pho*A gene was excised from pSS1324 (Table 3) and ligated into the *Xho*I site 3' to the *slt*-IIvhb A subunit and into the *Hind*III site within the polylinker of pSS1295. The resultant plasmid pSQ448 was transformed into DH5α and into O91:H21 EHEC strain B2F1, both of which were made competent for nucleic acid uptake by the method of Hanahan *et al.* (1983). Strains B2F1 and DH5α produce basal levels of alkaline phosphatase when grown in LB.

The amount of alkaline phosphatase produced by control strains and strains that contained pSQ448 was assessed by measuring alkaline phosphatase activity as described by Gutierrez *et al.* (1987) with the following modifications. An overnight culture was diluted 1:10 in 1 M Tris pH 8.0. The bacteria were then lysed by the addition of three drops of

Figure 3. Construction of *slt*-IIvhb A-*pho*A reporter gene fusion. The structural gene for alkaline phosphatase was placed under the transcriptional control of the *slt*-IIvhb A subunit promoter. So that translational regulation could also be assessed, the *pho*A gene was cloned in-frame to the toxin gene at a newly-constructed, unique *Xho*I restriction site.





phosphatase activity = SLT-IIvhb activity

toluene or by the addition of 0.1% SDS and $CHCl_3$ to the culture. When toluene was used as the lysing agent, it was evaporated from the lysate, and 0.4% Sigma 104 (in Tris pH 8.0) (Sigma Chemical Co.) was added. Sigma 104 is p-nitrophenol phosphate, a chromogenic substrate for alkaline phosphatase. When SDS and $CHCl_3$ were used to lyse the bacteria, the bacterial lysates were centrifuged before addition of the substrate. The reaction mixture was then agitated briefly and incubated at 37°C. The reaction was stopped with K_2HPO_4, and the intensity of the yellow color was read at an optical density of 420 nm. The units of alkaline phosphatase per milliliter culture were calculated as previously described (Gutierrez, *et al.*, 1987). The protein concentration of the lysates was determined with the Pierce BCA protein assay kit.

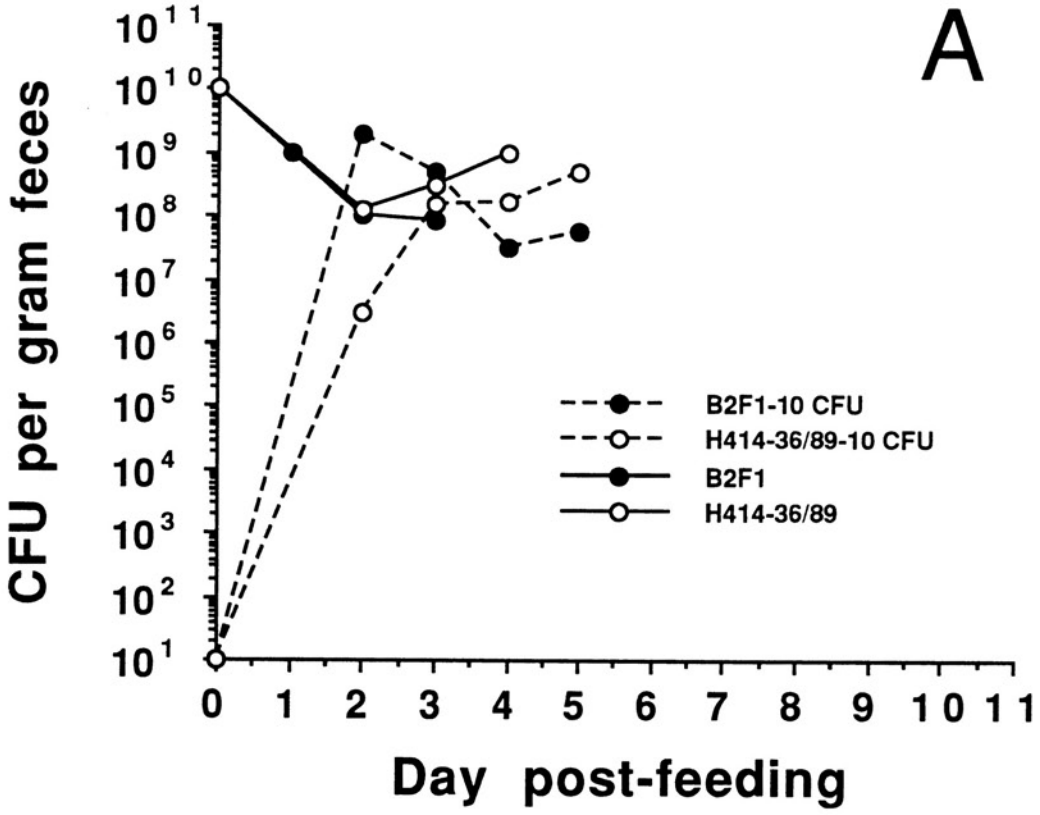
RESULTS

I. Virulence of SLT-II producing strains in a murine model for EHEC infection.

Virulence and colonization capacity in mice. In an earlier study from this laboratory, Wadolkowski *et al.* (1990a) observed that EHEC 0157:H7 strain 933 which makes SLT-I and SLT-II (Strockbine *et al.*, 1986) does not kill orally-challenged, streptomycin-treated mice. However, in that study, a variant of a plasmid-cured derivative of strain 933 (933cu-rev) was isolated that is lethal for streptomycin-treated mice at about 10^{10} CFU (Wadolkowski *et al.*, 1990a). 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 (Wadolkowski *et al.*, 1990b). In this project, the virulence of several clinical EHEC isolates that contained at least one toxin operon from the SLT-II group was assessed. Mice were treated with streptomycin and individually fed 10^{10} CFU of streptomycin resistant (Str^r) derivatives of 091:H21 strain B2F1, 091:H21 strain H414-36/89, 0157:H7 strain J-2, 0157:H- strain E32511 and its derivative E32511/HSC, or 0157:H7 strain 933 (as an avirulent control).

Mice fed the Str^r derivatives were monitored for intestinal colonization (Fig. 4) and death (Table 4).

Figure 4. Pattern of colonization of streptomycin-treated mice fed Str^r derivatives of (A) EHEC 091:H21 strains B2F1 or H414-36/89; (B) 0157:H- strains E32511, E32511/HSC, or 0157:H7 strain J-2. 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 feces of three mice from a single representative experiment. Colonization experiments were done one to five times for each strain. All of the mice fed 10^{10} CFU B2F1(Str^r) or H414-36/89(Str^r) died by day four post-feeding. Those mice fed 10 CFU of the 091:H21 strains died between day five and day eight post-feeding. Because each point represents three mice, error bars are not presented.


A
CFU per gram feces
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
0 1 2 3 4 5 6 7 8 9 10 11
Day post-feeding
B2F1-10 CFU
H414-36/89-10 CFU
B2F1
H414-36/89

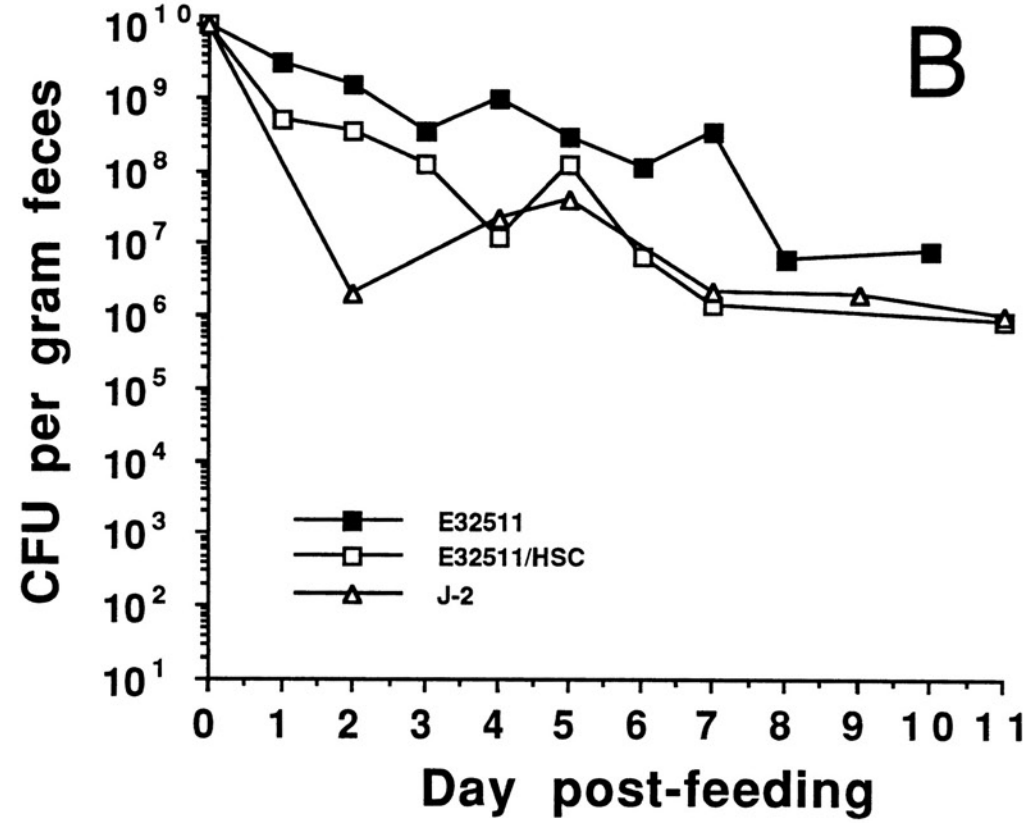

B
CFU per gram feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
0 1 2 3 4 5 6 7 8 9 10 11
Day post-feeding
E32511
E32511/HSC
J-2

Table 4. Virulence of EHEC strains for orally-inoculated, streptomycin-treated mice

EHEC strain fed	Mouse death[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)	0/8[d]	ND

[a] Number of mice that died per number orally-inoculated with 10^{10} CFU bacteria. Each ratio represents pooled data from three to six separate experiments.

[b] The LD_{50} was determined by the method of Reed and Muench (1938).

[c] ND, Not done because few or no mice in these groups died at oral doses of 10^{10} CFU.

[d] In addition to the data presented here, Wadolkowski *et al.* found that a total of 0/11 mice died when fed 10^{10} CFU of strain 933(Str^r) (Wadolkowski *et al.*, 1990a; Wadolkowski, unpublished results).

Mice in every test group were colonized with the infecting strain (Fig. 4). Strain 933 was previously shown to colonize CD-1 mice (Wadolkowski *et al.*, 1990a). All of the mice fed 10^{10} CFU B2F1(Str^r) or H414-36/89(Str^r) died between day three and day five post-feeding (Table 4); therefore, strains B2F1 and H414 were classified as highly virulent in this mouse model. Occasionally a mouse fed strain E32511(Str^r) or E32511/HSC(Str^r) died between day five and day twenty-three post-feeding (Table 4). Passage of strain E32511(Str^r) or E32511/HSC(Str^r) through mice did not increase the pathogenicity of these isolates; all of the mice fed mouse-passaged isolates of strain E32511(Str^r) (10/10) or E32511/HSC(Str^r) (10/10) survived. One mouse out of nineteen fed strain J-2(Str^r) died, and strain 933(Str^r) was avirulent in the mouse model (Table 4).

To verify the assumption that streptomycin-resistance did not affect the virulence of the EHEC strains, the cytotoxicity and *in vitro* growth rates of the Str^r derivatives were compared with the wild-type (wt) strains. The cytotoxic titers (CD_{50}/ml culture) of the EHEC Str^r derivatives isolated for this study were similar to the wild-type strains: B2F1(Str^r), 5 x 10^2, B2F1-wt, 5 X 10^2; H414-36/89(Str^r), 1 X 10^6, H414-36/89-wt, 1 X 10^6; E32511(Str^r), 1 x 10^5, E32511-wt, 5 X 10^5; E32511/HSC(Str^r), 1 X 10^3, E32511/HSC-wt, 1 X 10^3; J-2(Str^r), 1 X 10^5, J-2-wt 1 X 10^5. The *in vitro* growth rates of the Str^r derivatives were also similar to the

parental strains (Fig. 5). To determine whether streptomycin affected the bacterial growth rate, the *in vitro* growth rates of strain B2F1(Str^r) and strain H414-36/89(Str^r) were examined in the presence and absence of streptomycin. No differences in growth rates were observed (Fig. 6). *E. coli* strain 933(Str^r) was previously shown to have comparable growth rate and cytotoxic titer as the wild-type 933 strain (Wadolkowski *et al.*, 1990a).

Colonization of EHEC strains in non-streptomycin treated mice. Myhal *et al.* (1982) has demonstrated that streptomycin treatment suppresses the level of normal bacterial flora in the mouse intestine. To determine whether streptomycin treatment was required for colonization and virulence of B2F1(Str^r) or H414-36/89(Str^r), a dose of 10^{10} CFU of the highly virulent strain B2F1(Str^r) or H414-36/89(Str^r) was fed to non-streptomycin treated mice. By day four post-inoculation, the level of CFU/g feces of mice fed either of these 091:H21 strains had dropped to approximately 5 x 10^4 CFU/g feces or lower (Fig. 7). Furthermore, by day 8 post-infection, both EHEC strains were present in the feces at close to or below the level of detection (10^2 CFU/g 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 091:H21 strains B2F1(Str^r) or H414-36/89(Str^r).

Figure 5. *In vitro* growth rate of (A) B2F1(Str^r), (B) H414-36/89(Str^r), (C) E32511(Str^r), and (D) E32511/HSC(Str^r), and the respective parent EHEC strain. A 1:100 dilution of a saturated overnight culture was made in LB broth. The optical density at 600 nm of each culture was measured approximately every 30 min until the cultures reached stationary growth phase.

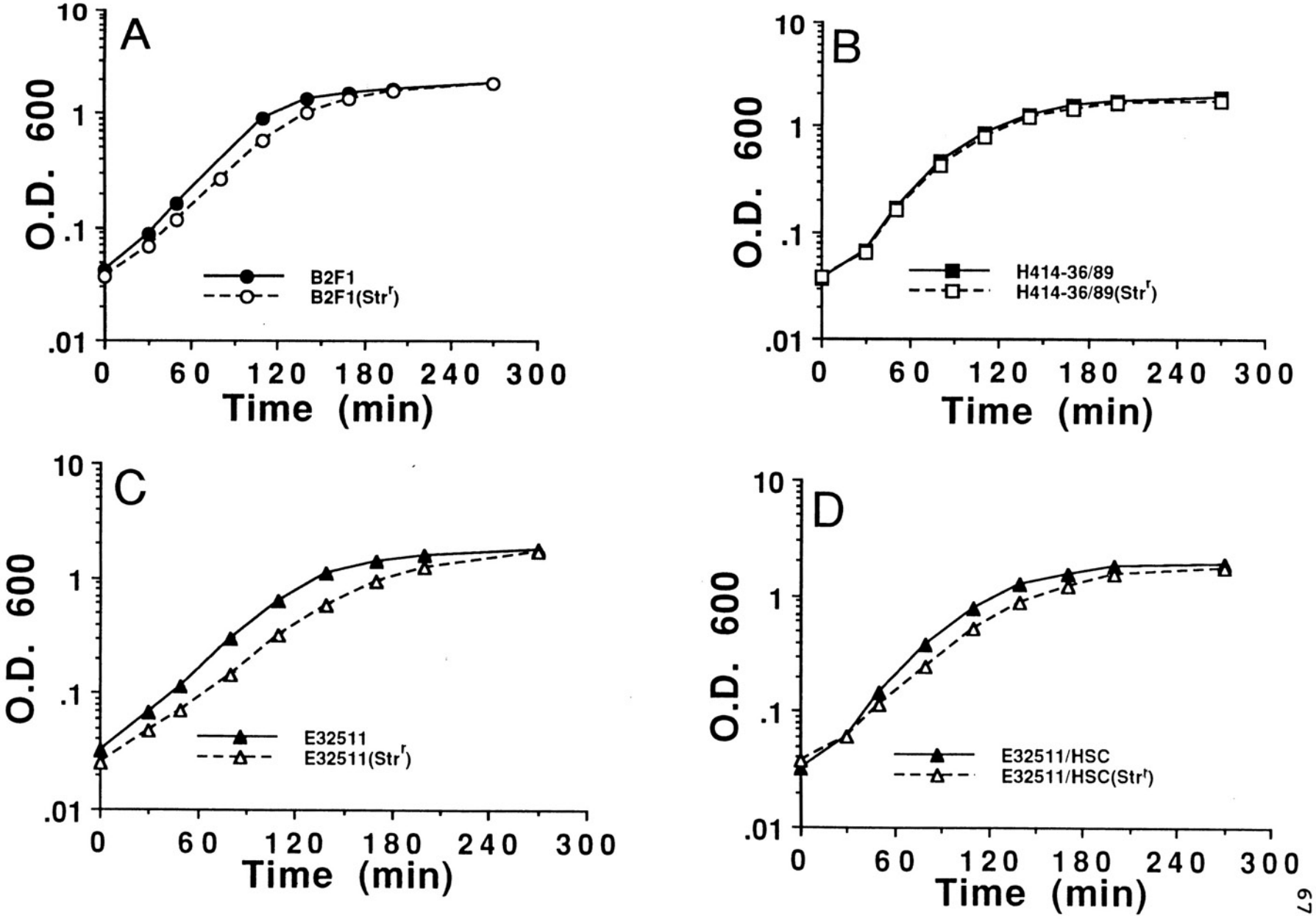

A
O.D. 600
10
1
.1
.01
0
60
120
180
240
300
Time (min)
B2F1
B2F1(Str^r)
B
O.D. 600
10
1
.1
.01
0
60
120
180
240
300
Time (min)
H414-36/89
H414-36/89(Str^r)
C
O.D. 600
10
1
.1
.01
0
60
120
180
240
300
Time (min)
E32511
E32511(Str^r)
D
O.D. 600
10
1
.1
.01
0
60
120
180
240
300
Time (min)
E32511/HSC
E32511/HSC(Str^r)

Figure 6. *In vitro* growth rate of 091:H21 strain B2F1(Str^r) and H414-36/89(Str^r) in the presence of 20 μg/ml streptomycin. A 1:100 dilution of saturated overnight cultures of B2F1(Str^r) and H414-36/89(Str^r) was made in LB broth and LB supplemented with streptomycin. The optical density at 600 nm of each culture was measured approximately every 30 min until the bacteria reached stationary growth phase.

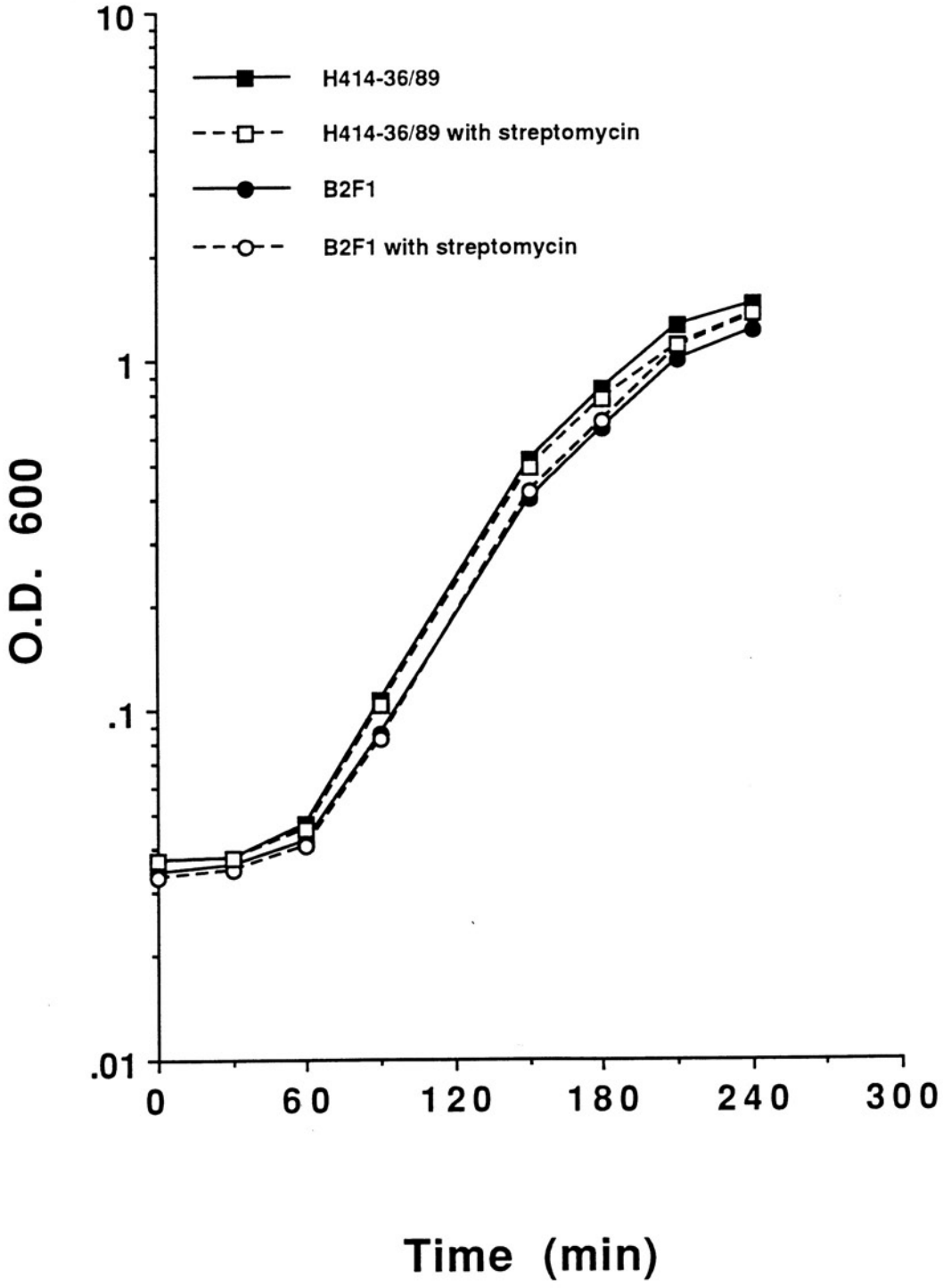
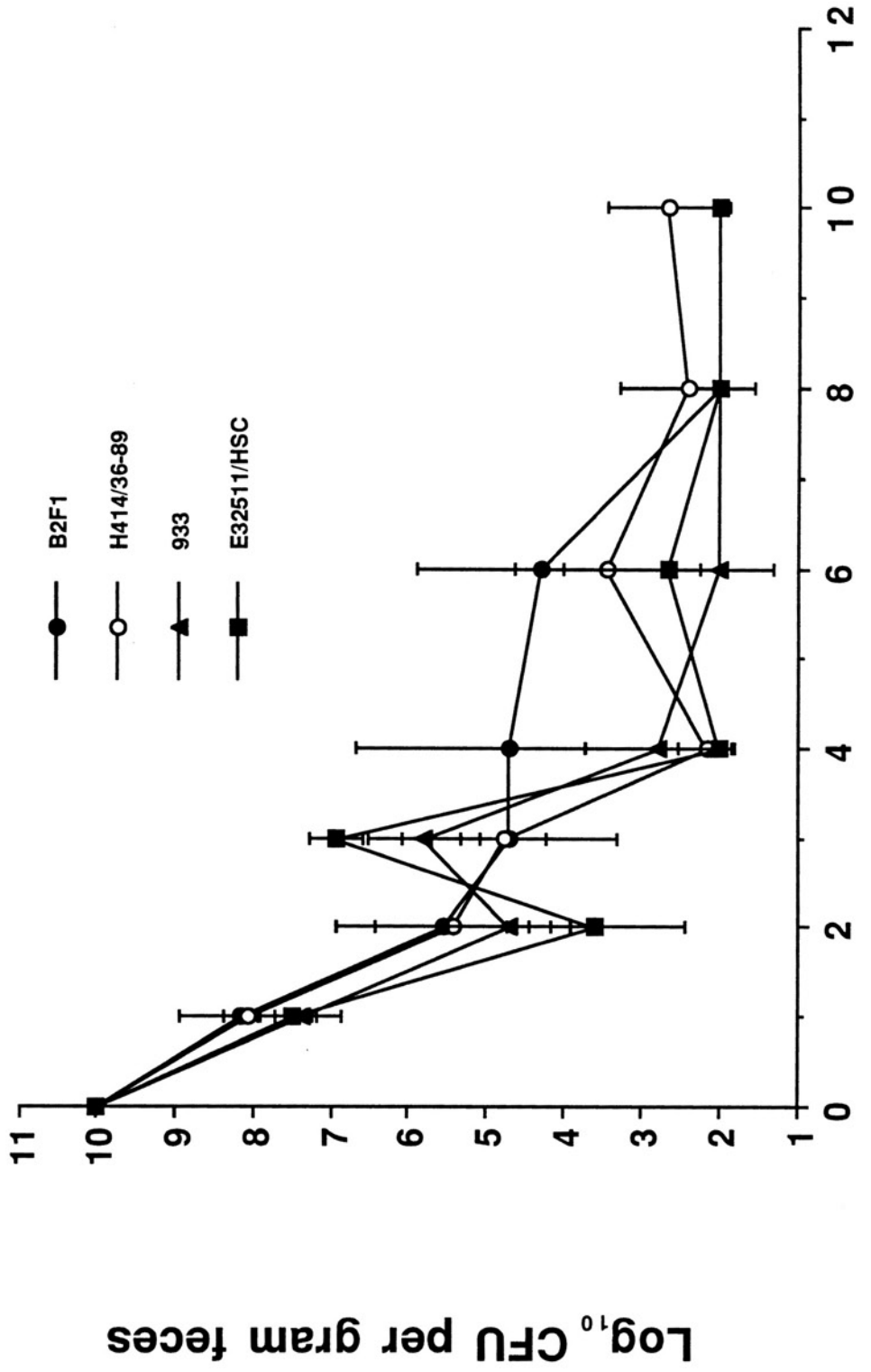

10
1
.1
.01
O.D. 600
H414-36/89
H414-36/89 with streptomycin
B2F1
B2F1 with streptomycin
0
60
120
180
240
300
Time (min)

Figure 7. 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 10^2 CFU/g feces. Each point represents the geometric mean CFU per gram feces from four mice. The error bars represent plus or minus two standard errors of the geometric mean.


B2F1
H414/36-89
933
E32511/HSC
Log10 CFU per gram feces
Day post-feeding
11
10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12

Plasmid analysis of EHEC strains. Previous data from our laboratory suggest that the 60MDa EHEC plasmid augments the colonizing capacity of O157:H7 strains in streptomycin-treated mice (Wadolkowski *et al.*, 1990a). Therefore, the question of whether mouse-virulent O91:H21 strains contain such a plasmid was addressed. First, the plasmid profiles of strains B2F1 and H414-36/89 were compared to those of O157 strains E32511, E32511/HSC, 933, and 933cu. All the strains tested except 933cu appeared to have a large plasmid (Fig. 8). Next, the same set of strains were probed with a 3.4 kb *Hind*III fragment of the EHEC plasmid (Levine *et al.*, 1987) to verify that the large plasmid in the O91:H21 strains was indeed a plasmid related to the EHEC large plasmid. A 3.4 kb *Hind*III fragment in strain B2F1, H414-36/89, E32511, E32511/HSC, and 933 (as a control) hybridized with the EHEC plasmid probe (Fig. 9). Plasmid DNA from the negative control, 933cu, did not hybridize with the probe (Fig. 9). Although the presence of additional hybridized bands suggests incomplete DNA digestion, these data demonstrate that both of the O91:H21 strains contain a plasmid of appropriate size with sequences homologous to the EHEC plasmid from which the probe was derived.

Oral LD_{50} of the O91:H21 EHEC strains. To assess the oral LD_{50} of the highly virulent strains, groups of five streptomycin-

Figure 8. Plasmid profiles of EHEC strains 933, 933cu, B2F1, H414-36/89, E32511, and E32511/HSC. Arrow indicates size of large plasmid present in strain 933. Large plasmids of a similar size were present in all of the other strains except strain 933cu. Molecular weight markers were lambda *Hind*III fragments of the sizes shown (kD).

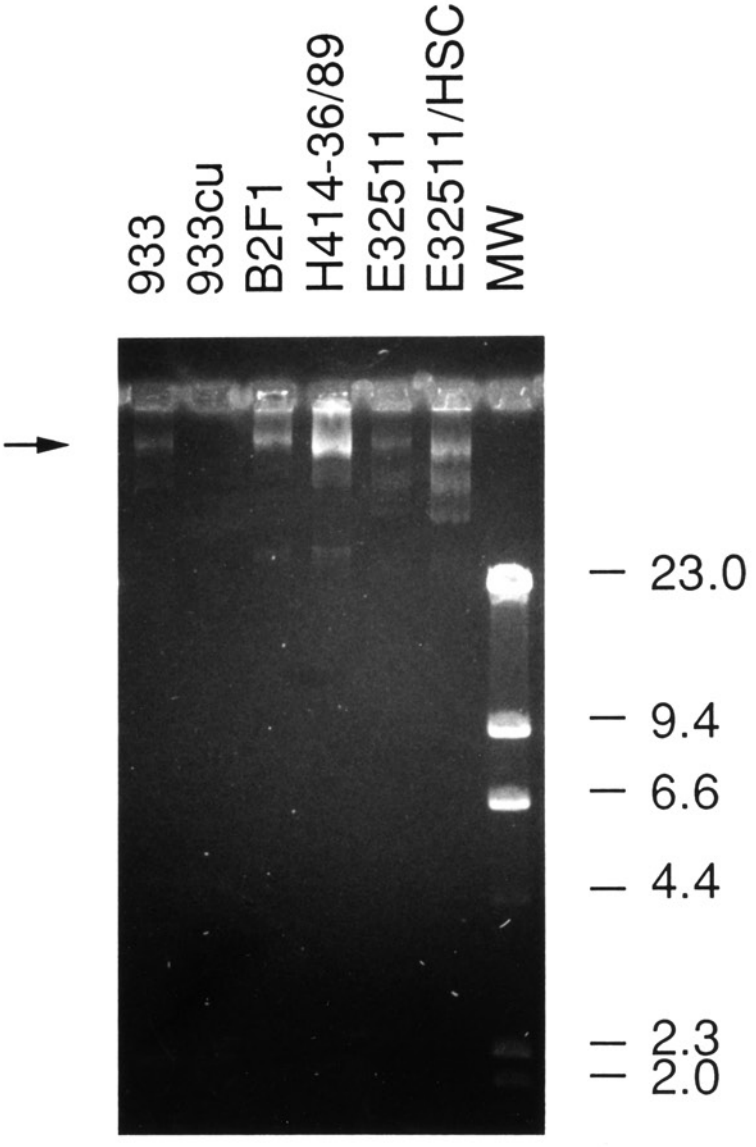
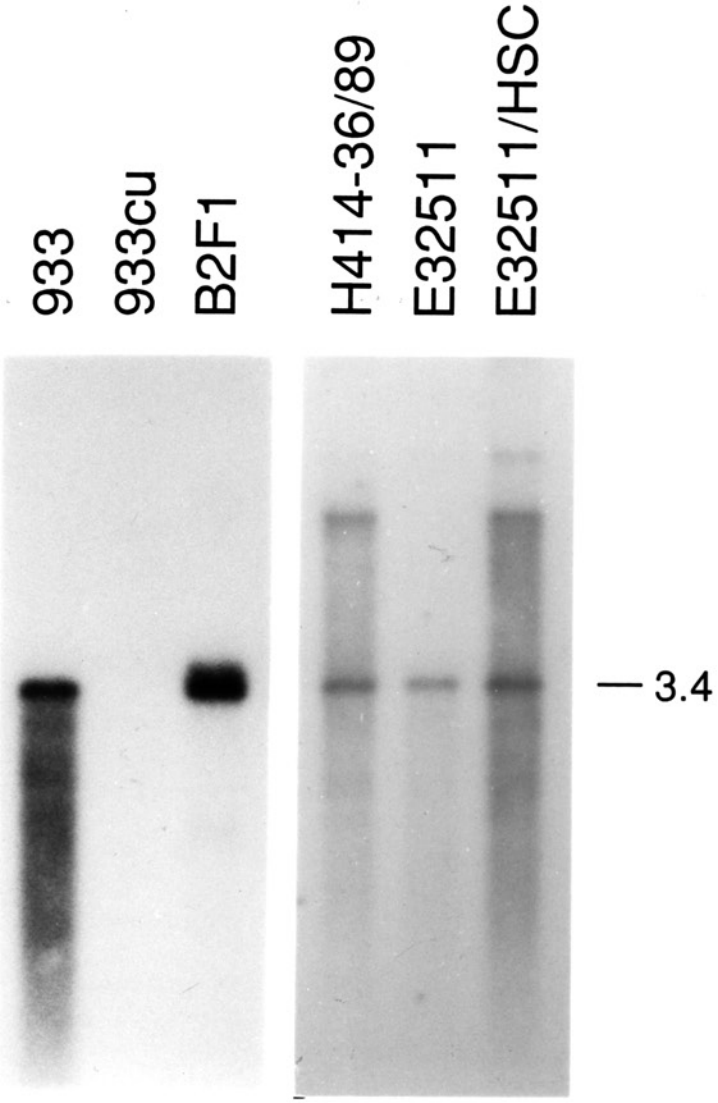

933
933cu
B2F1
H414-36/89
E32511
E32511/HSC
MW
23.0
9.4
6.6
4.4
2.3
2.0

Figure 9. *In situ* gel hybridization of plasmid DNA from strains 933, 933cu, B2F1, H414-36/89, E32511, and E32511/HSC digested with *Hind*III and hybridized with a 3.4 kb EHEC plasmid DNA probe.


933
933cu
B2F1
H414-36/89
E32511
E32511/HSC
3.4

treated mice were fed dilutions from 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} for both B2F1(Str^r) and H414-36/89(Str^r) was less than 10^1 CFU (Table 4). 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 to 10^9 CFU/g feces (Fig. 4). These animals died within five- to eight- days post-feeding.

Bacteriological and histological examination of moribund mice. No Str^r bacteria were found in blood, homogenized spleens, or kidneys of B2F1(Str^r)-infected mice as indicated by bacterial plate counts. This observation indicates that B2F1 is not invasive. To determine the cause of death of the mice infected with strain B2F1(Str^r), moribund animals were sacrificed and a variety of tissues were examined histologically. No lesions were observed in any section except those from the kidneys where renal tubular dilation, degeneration, and necrosis were observed (normal kidney, Fig. 10; damaged kidney, Fig. 11). Extensive renal tubular necrosis was also seen in the kidneys of moribund mice infected with *E. coli* strain H414-36/89(Str^r) (Fig. 12). These histological results suggest that the mice fed either

Figure 10. Photomicrograph of the renal cortex from a normal CD-1 mouse. No lesions were evident in the renal cortex of this animal. Hematoxylin and eosin; magnification, X200.

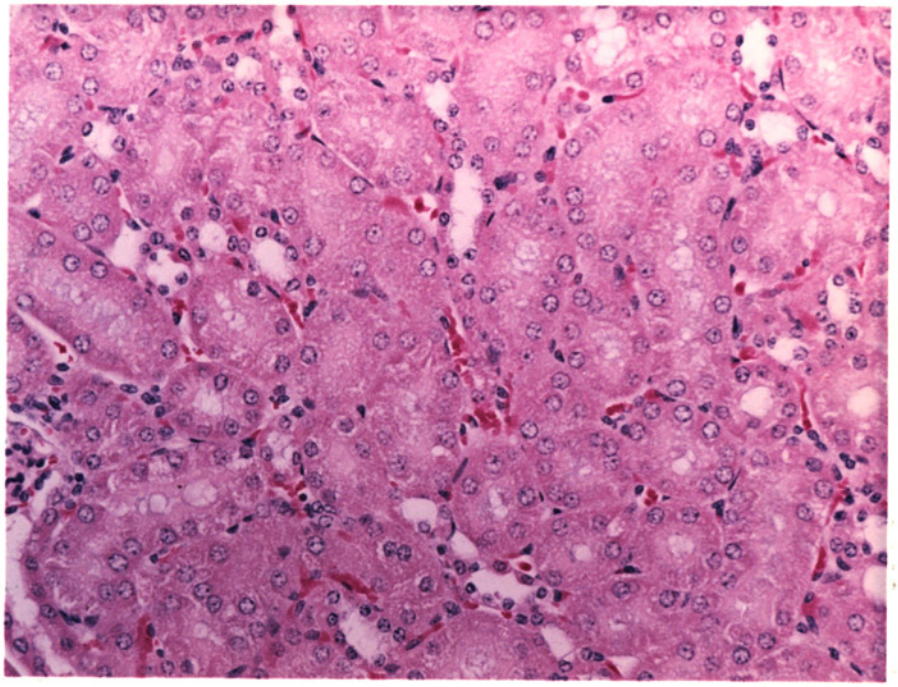
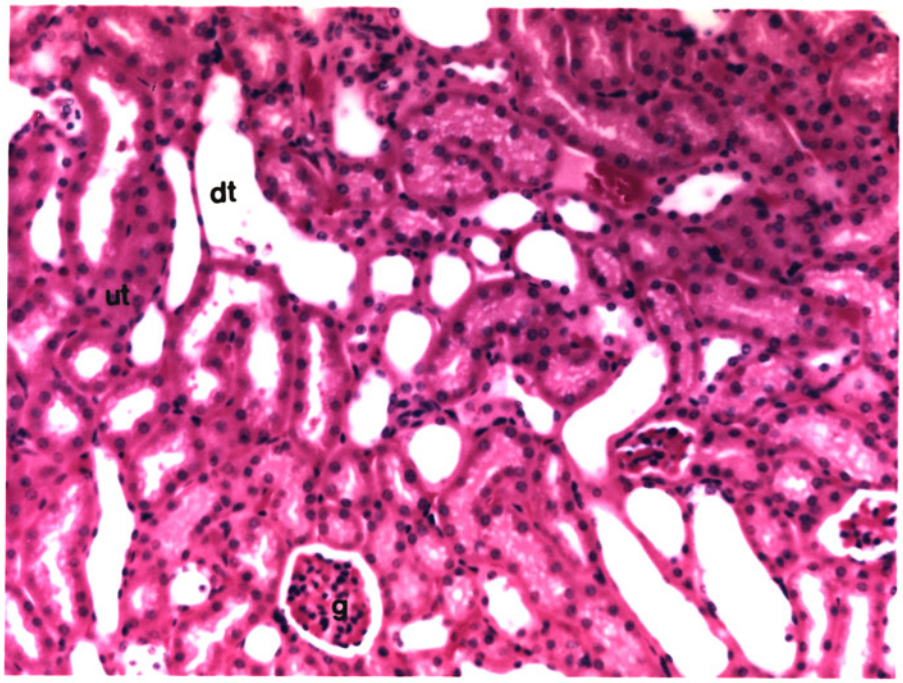


Figure 11. Photomicrograph of renal cortex (glomeruli, tubules) from a moribund, streptomycin-treated, CD-1 mouse fed 10^{10} CFU *E. coli* strain B2F1(Str^r). The mouse was sacrificed on day three post-feeding, and the tissue was prepared as described in Materials and Methods. The lumens of many of the tubules were dilated and contained necrotic tubular epithelial cells. The dilated tubules (dt) were lined with fewer, more flattened, tubular epithelial cells than the lumens of unaffected tubules (ut) due to loss of the adjacent cells. The glomeruli (g) were histologically normal. Hematoxylin and eosin; magnification, X200 (top), X400 (bottom).


dt
ut
g

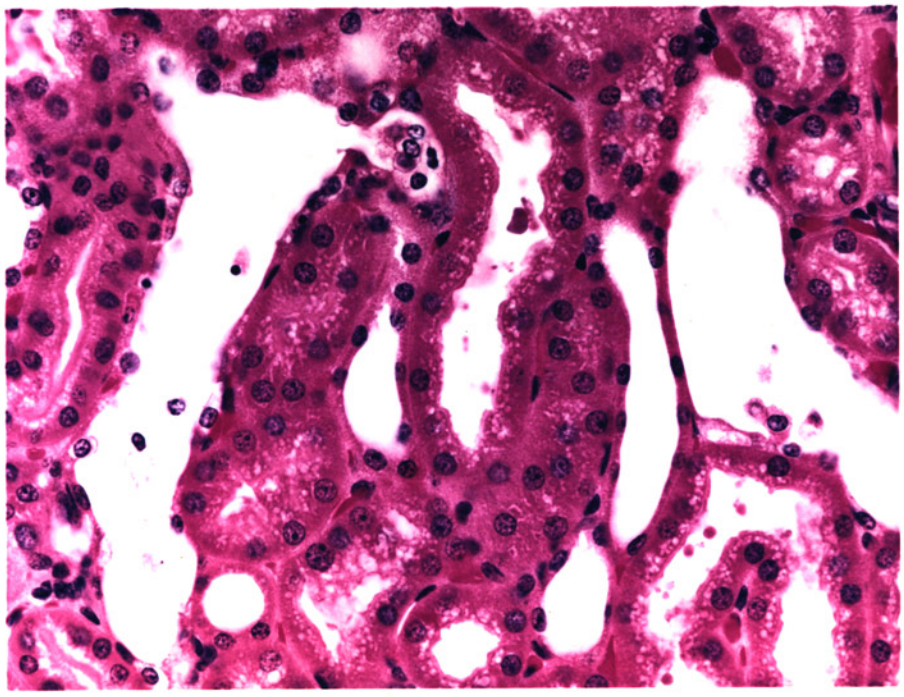
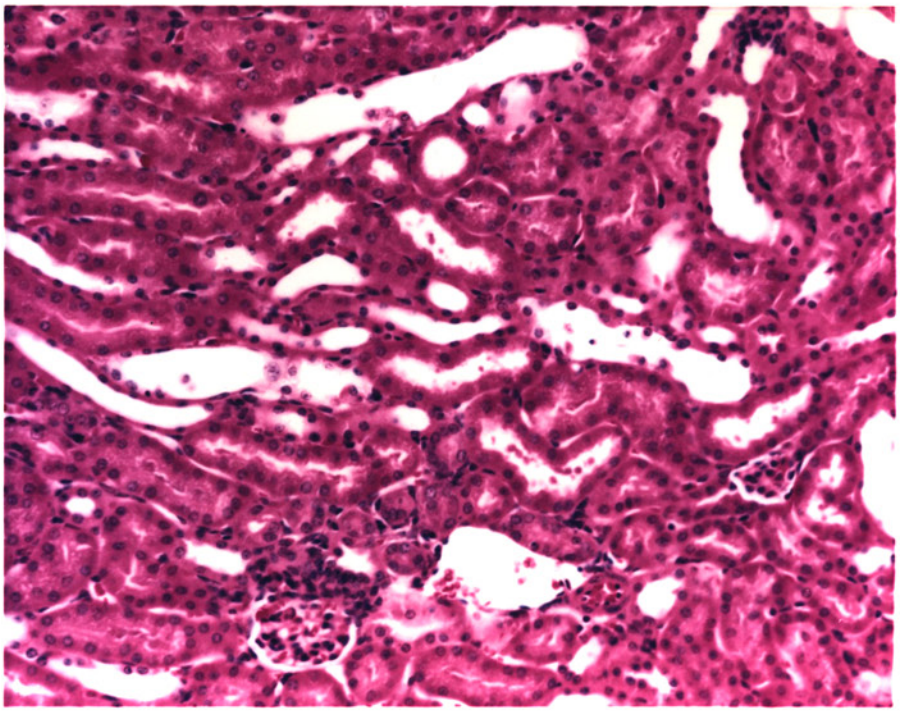
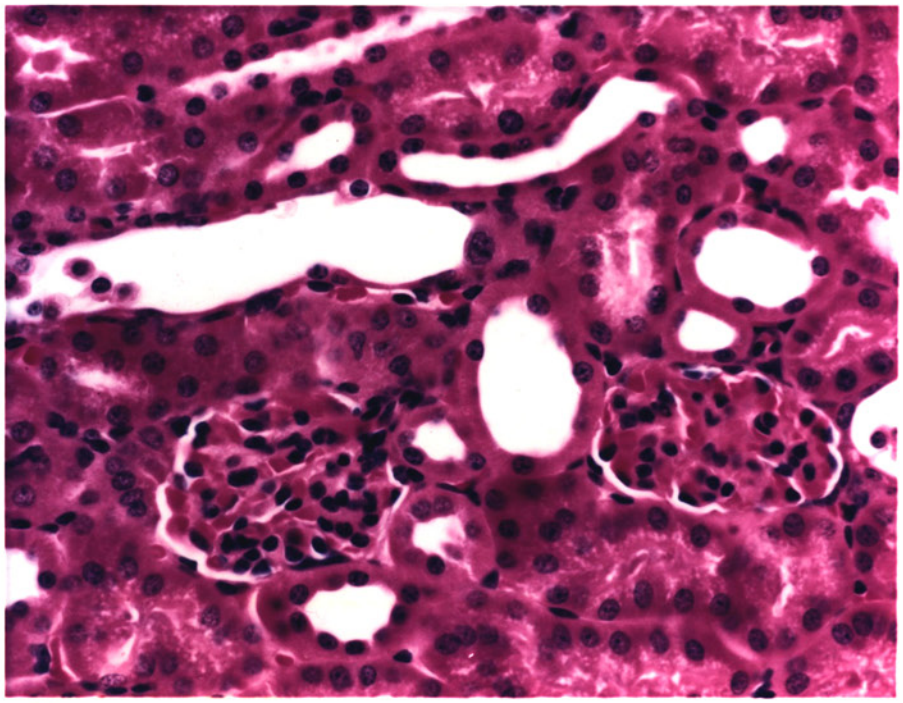


Figure 12. Photomicrograph of renal cortex from a moribund, streptomycin-treated CD-1 mouse fed 10^{10} CFU of EHEC strain H414-36/89(Str^r). The mouse was sacrificed on day three post-feeding, and the tissue was prepared as described in Materials and Methods. Acute cortical tubular necrosis and severe dilation of tubular lumens was observed. The glomeruli were histologically normal, and there was no disruption of the tubular basement membrane. Hematoxylin and eosin; magnification, X200 (top), X400 (bottom).





B2F1(Str^r) or H414-36/89(Str^r) died of renal tubular necrosis, a conclusion that is consistent with previous findings from mice fed 933cu-rev (Wadolkowski *et al.*, 1990a).

Hematological examination of mice infected with 091:H21 strain B2F1. As mentioned previously, some patients infected with EHEC develop HUS. Hallmarks of EHEC HUS include renal glomerular damage, thrombocytopenia, and hemolytic anemia (Karmali, 1989). 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 5). Although the blood chemistry and hematological results were variable, slight increases in hemoglobin, hematocrit, and platelet counts were noted. The hematological test results obtained were not characteristic of HUS (Ratnam *et al.*, 1985; Neill *et al.*, 1987) and may indicate hemoconcentration. The BUN and creatinine values were elevated when compared to 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.

Footnotes for Table 5

[a] Values given are the mean ± SD; n=6.

[b] Mice were orally inoculated with 10^6 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 6-8 week old mice (n=20).

Table 5. Hematology and Blood Chemistry Results of B2F1(Str^r)-infected and Uninfected Streptomycin-treated CD-1 Mice

Mouse group[a]	Test				
	Hemoglobin (g/dl)	Platelets (x $10^3/mm^3$)	Hematocrit (%)	BUN (mg/dl)	Creatinine (mg/dl)
Streptomycin-treated & B2F1(Str^r) inoculated[b]	18.5 ± 0.6	2238 ± 707	53 ± 4	79 ± 33	0.7 ± 0.6
Streptomycin treated and uninfected	14.6 ± 1.0	1489 ± 174	43 ± 2	34 ± 6	0.2 ± 0.1
Untreated and uninfected[c]	14.4 ± 0.9	1200 ± 100	42 ± 3	33 ± 5	0.5 ± 0.2

II. Analysis of 091:H21 *E. coli* virulence in mice.

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 strain H414-36/89(Str^r). Administration of anti-SLT-II B subunit antibody did not appear to affect bacterial colonization (Fig. 13) but did protect the mice from the lethal effects of both B2F1(Str^r) and H414-26/89(Str^r) for greater than 30 days (Table 6). By contrast, neither the control anti-SLT-I B subunit monoclonal nor the PBS control prevented death of the infected mice (Table 6). Furthermore, no kidney damage was observed in selected anti-SLT-II B subunit antibody-treated, infected mice (Fig. 14). Taken together, these results indicate that SLT-II-related toxins play a pivotal role in the death of the orally-infected, streptomycin-treated mice.

***In vivo* colonization characteristics of strain 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 strains B2F1 and H414-36/89 may either express more toxin *in vivo* than the other strains examined or they may have

Figure 13. Colonization of passively-immunized, B2F1(Str^r)- and H414-36/89(Str^r)-inoculated CD-1 mice. Each point represents the geometric mean CFU per gram feces from three individually-housed mice. The data from the B2F1(Str^r)-inoculated mice were taken from one of two experiments. The data from the H414-36/89(Str^r)-inoculated mice were taken from a separate experiment. The majority of the mice passively immunized survived; all of the mice that were not passively immunized died. Because the sample size for this experiment was three, error bars are not presented. The range of CFU values for mice within a passively immunized group overlapped the range of CFU values from the corresponding non-immunized mouse group.

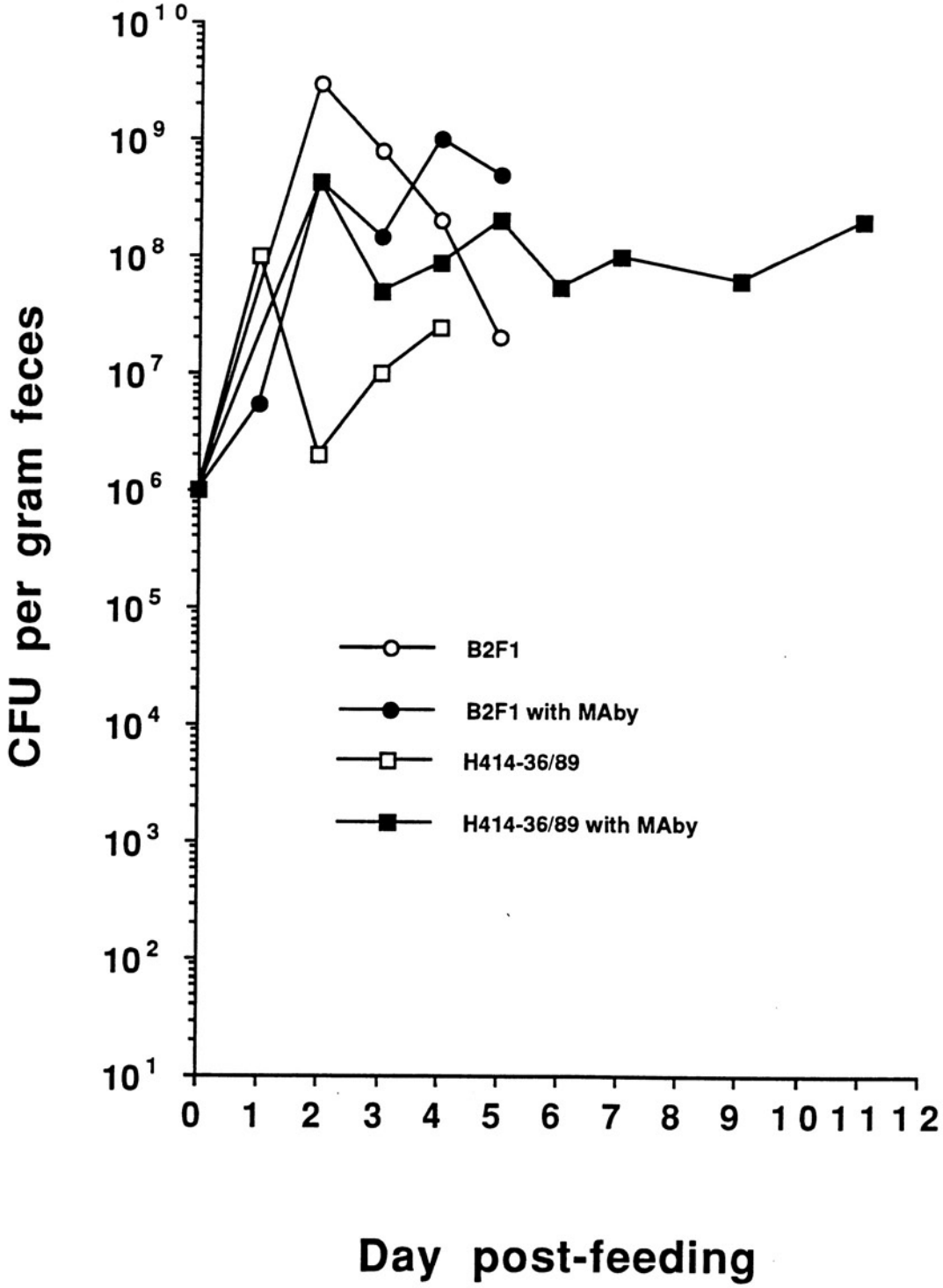
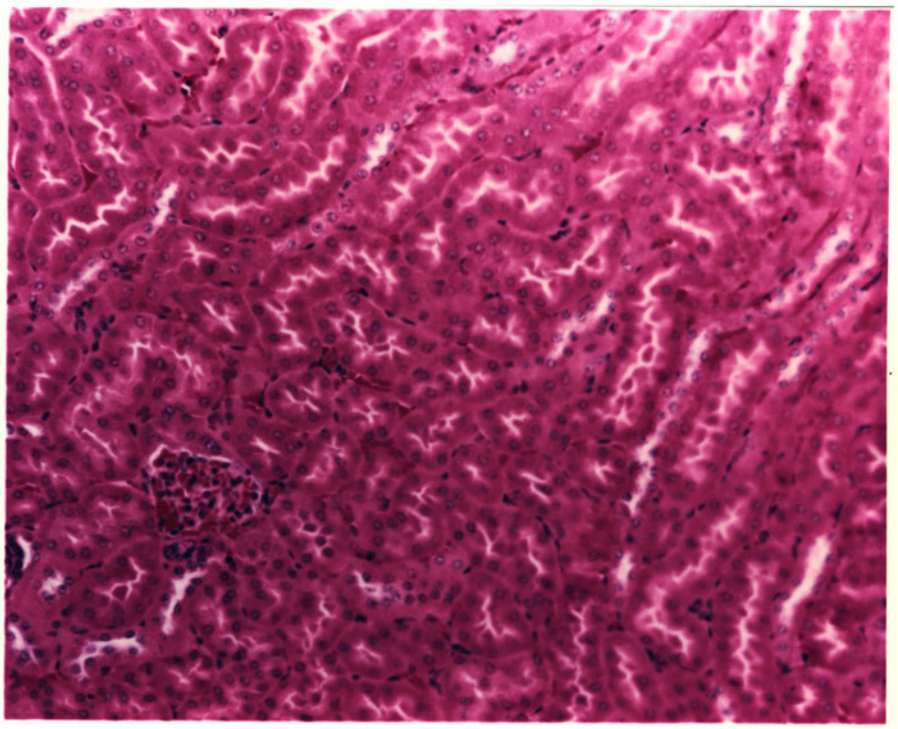

CFU per gram feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
0 1 2 3 4 5 6 7 8 9 10 11 12
Day post-feeding
B2F1
B2F1 with MAby
H414-36/89
H414-36/89 with MAby

Table 6. Passive Immunization of Streptomycin-treated CD-1 Mice Infected with E. coli 091:H21 Strain B2F1(Str^r) or H414-36/89(Str^r)

Monoclonal Antibody		Mouse survival[a]	
Designation	Directed against	B2F1(Str^r) infected	H414-36/89(Str^r) infected
BC5BB12	SLT-II B subunit	10/10	5/5
13C4	SLT-I B subunit	1/10	0/5
PBS control	-	1/10	0/5

[a] Number of animals remaining alive on day 30 post-feeding per number initially infected. Mice were inoculated i.p. with 1 ml of a 1:1000 dilution of ascites in PBS or PBS alone both 24 hours and immediately prior to feeding 10^6 CFU of bacteria. Experiments were done three times for B2F1(Str^r) 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.

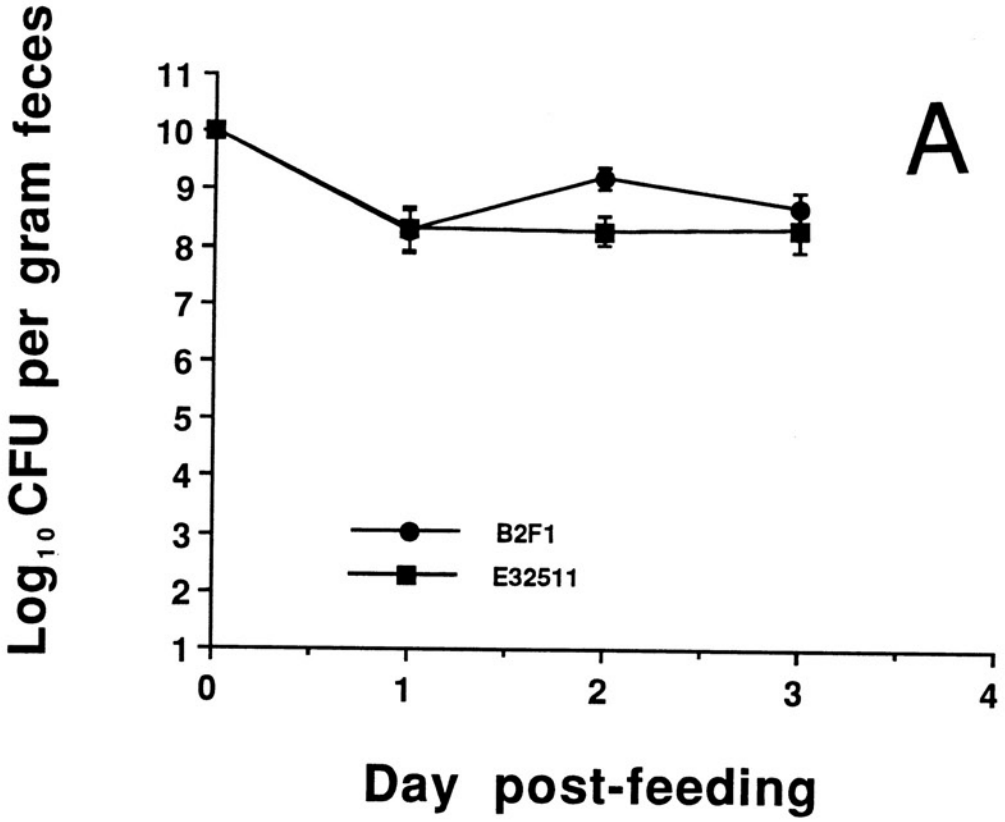
Figure 14. Photomicrograph of renal cortex from passively-immunized mouse inoculated with 10^6 CFU of EHEC strain B2F1(Str^r). The mouse was sacrificed forty days post-feeding. Signs of illness were not apparent. The renal cortex was histologically normal. Hematoxylin and eosin; magnification, 200X.

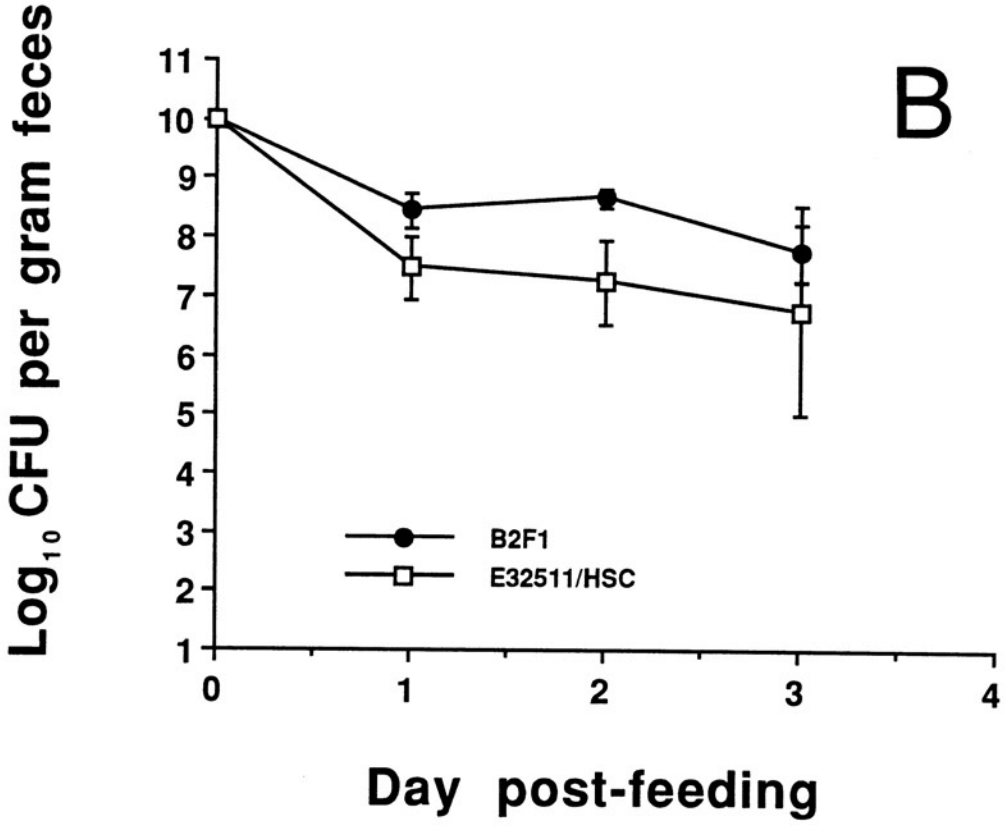


additional virulence factors that augment toxin delivery to the mouse. To determine whether B2F1(Str^r) and H414-36/89(Str^r) are able to colonize the mouse intestine better than the other EHEC strains tested, several parameters of colonization were examined. First, the capacity of selected strains to colonize the mouse intestine in the presence of normal flora was determined. The data shown in Figure 7 indicate that strain 933(Str^r) and strain E32511/HSC(Str^r) were as efficient as highly virulent B2F1(Str^r) and H414-36/89(Str^r) in colonizing the normal mouse intestine. Second, the capacity of the lethal EHEC strain B2F1 to compete with either strain E32511 or strain E32511/HSC for intestinal colonization was evaluated. When 10^{10} CFU of strain B2F1(Str^r, Nal^r) and 10^{10} CFU of strain E32511(Str^r, Rif^r) were fed simultaneously to mice, both strains persisted at levels of 10^8-10^9 CFU/g feces until day four when all of the mice died (Fig. 15A). Similar results were obtained when mice were co-fed 10^{10} CFU of strain B2F1(Str^r, Nal^r) and 10^{10} CFU of strain E32511/HSC(Str^r, Rif^r) (Fig. 15B). These co-colonization results suggest that either these EHEC 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 investigated was whether B2F1(Str^r) or H414-36/89(Str^r) colonize the mouse small intestine. Wadolkowski *et al.* (1990a) previously showed that

Figure 15. Co-colonization pattern of streptomycin-treated mice fed (A) 10^{10} CFU each of strain B2F1(Str^r, Nal^r) and strain E32511(Str^r, Rif^r); or (B) 10^{10} CFU each of strain B2F1(Str^r, Nal^r) and strain E32511/HSC(Str^r, Rif^r). At the times indicated, fecal samples were plated on MacConkey agar that contained streptomycin and rifampin and also on MacConkey agar that contained streptomycin and nalidixic acid. Each point represents the geometric mean CFU per gram feces from four mice. The error bars represent plus or minus two standard errors of the geometric mean.


A
Log10 CFU per gram feces
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
B2F1
E32511
Day post-feeding


B
Log10 CFU per gram feces
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
B2F1
E32511/HSC
Day post-feeding

the mouse virulent EHEC strain 933cu-rev, unlike its mouse avirulent parent 933cu or 933, could colonize the small as well as large intestine at high levels. To define the location of B2F1(Str^r) and H414-36/89(Str^r) colonization, the number of lactose-positive, Str^r bacteria present in intestinal epithelial cell preparations from various intestinal segments of infected mice was measured (Fig. 16). It should be noted that bacteria were not removed from the epithelial cells prior to plating. Therefore, one epithelial cell with several adherent bacteria may be represented by only one CFU. No significant differences in the number of Str^r bacteria present in the small intestinal epithelial cell preparations were observed between virulent strains B2F1 and H414-36/89 and the avirulent control 933 (Fig. 16). To determine the total number of Str^r EHEC bacteria located in the intestinal mucosa, intestinal segments were isolated from mice orally-infected with virulent and avirulent EHEC strains and homogenized as described in Materials and Methods. Data from the first such experiment indicated that 091:H21 strains B2F1(Str^r) and H414-36/89(Str^r) were present at significantly higher levels in the proximal and mid-small intestine than strain 933(Str^r) (Fig. 17A). However, when this location of colonization experiment was repeated to include strain E32511/HSC(Str^r), the numbers of bacterial CFU in the small intestine were approximately the same for all EHEC strains tested (Fig. 17B). In the experiments described here,

Figure 16. Number of EHEC CFU found associated with isolated epithelial cells from the following segments of the intestine: small proximal (sp), small mid (sm), small distal (sd), cecum, large proximal (lp), and large distal (ld). *E. coli* strain B2F1(Str^r), H414-36/89(Str^r), or 933(Str^r) (10^{10} CFU) were fed to five streptomycin-treated CD-1 mice. The animals were sacrificed and intestinal epithelial cells were isolated as described in Materials and Methods. The bar at each point represents two standard errors of the geometric mean CFU per intestinal segment.

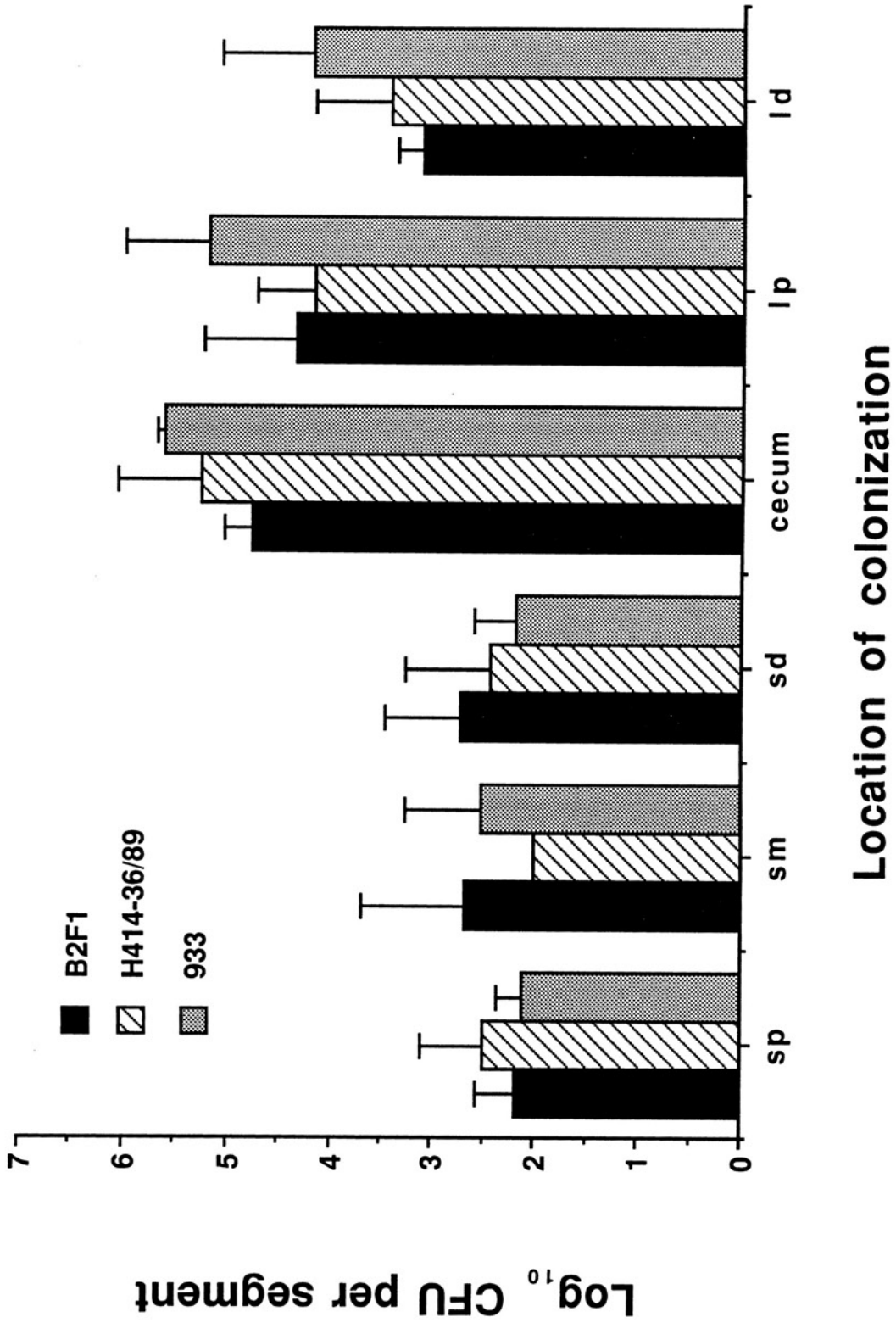
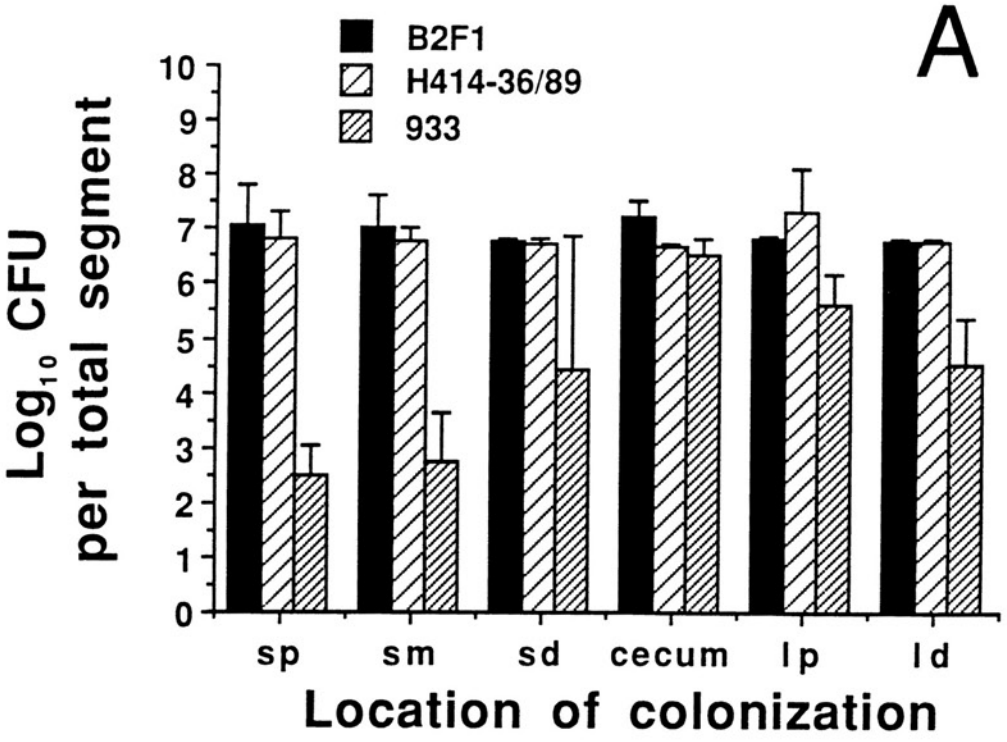
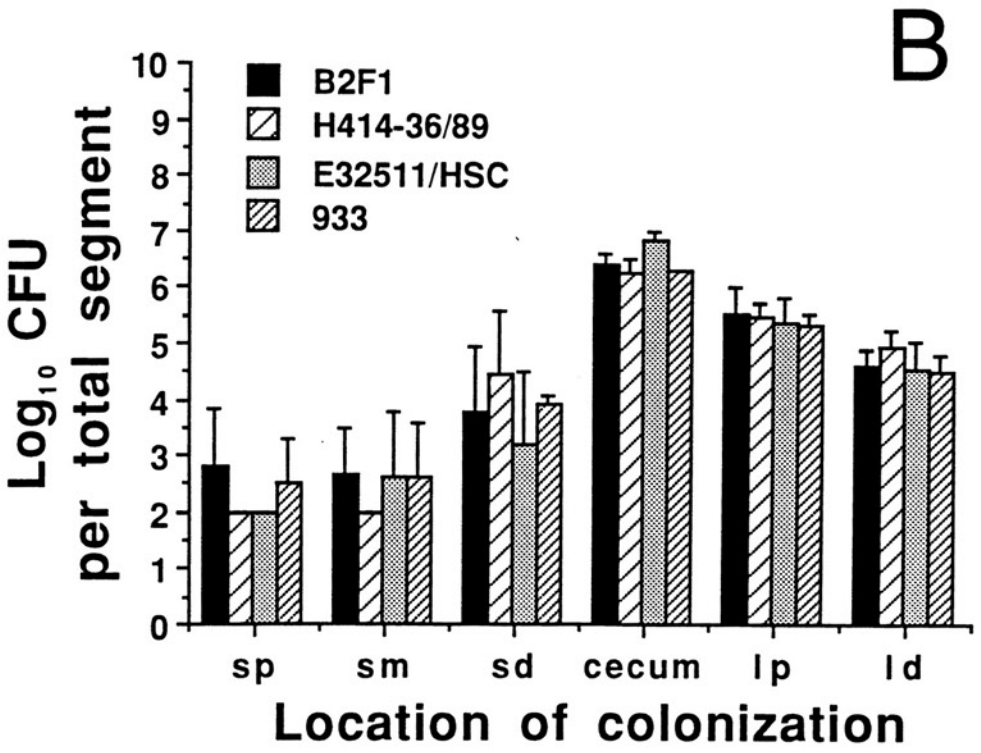

B2F1
H414-36/89
933
Log10 CFU per segment
0
1
2
3
4
5
6
7
sp
sm
sd
cecum
lp
ld
Location of colonization

Figure 17. Number of EHEC CFU present in homogenized intestinal segments from the small proximal (sp), small mid (sm), small distal (sd), cecum, large proximal (lp), and large distal (ld) regions. *E. coli* strain B2F1(Str^r), H414-36/89(Str^r), E32511/HSC(Str^r), or 933(Str^r) (10^{10} CFU) were fed to five streptomycin-treated mice. Data from experiment #1 (panel A), and experiment #2 (panel B) are presented. The animals were sacrificed and intestinal segments were isolated, washed, homogenized, and plated on MacConkey agar as described in Materials and Methods. The bar at each point indicates two standard errors of the geometric mean CFU per intestinal segment.


A
B2F1
H414-36/89
933
Log10 CFU per total segment
0
1
2
3
4
5
6
7
8
9
10
sp
sm
sd
cecum
lp
ld
Location of colonization


B
B2F1
H414-36/89
E32511/HSC
933
Log10 CFU per total segment
0
1
2
3
4
5
6
7
8
9
10
sp
sm
sd
cecum
lp
ld
Location of colonization

B2F1(Str^r) and H414-36/89(Str^r) were occasionally, but not consistently, found in high numbers in the small intestine. The inconsistency of bacterial counts in the small intestinal segments may have been due, in part, to experimental manipulations. Specifically, the bacteria could have been removed by the initial washing of the 3 cm segments if the organisms actively grew in the mucosa but did not firmly adhere to the intestinal epithelial cells.

III. Isolation and characterization of toxins produced by EHEC strains B2F1 and H414-36/89.

Shiga-like toxin gene profile. Previous DNA colony blot and toxin neutralization results identified H414-36/89 as an SLT-II producer (Bockemuhl *et al.*, 1992). To further characterize this highly mouse-virulent strain, the number of *slt*-II genes present was determined. 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 *Eco*RI or *Hind*III because neither of these restriction enzymes cut within genes from the *slt*-II toxin group (Jackson *et al.*, 1987; Weinstein *et al.*, 1988b; Ito *et al.*, 1991; Schmitt *et al.*, 1991). *In situ* gel hybridization with an *slt*-II probe resulted in the predicted number of fragments: B2F1 (2), E32511 (2), and

E32511/HSC (1). However, H414-36/89 had three toxin gene fragments (Fig 18).

Cloning of B2F1 and H414-36/89 toxin operons. The passive immunization results indicated that SLT-II toxin production is critical for the death of orally-infected mice. However, the relationship, if any, between the *slt*-II type and toxin operon copy number and the virulence of an EHEC strain in the orally-infected, streptomycin-treated mouse model was not clear. To begin to clarify these issues, the toxin operons from strain B2F1 and H414-36/89 were cloned. The *slt*-IIvha operon was isolated from a B2F1 cosmid bank as described in Materials and Methods. Figure 19 is an *in situ* gel hybridization of total DNA from strain B2F1 and plasmid DNA from the two isolated toxin cosmid clones digested with *Pst*I and hybridized with an *slt*-II probe. The *slt*-IIvha and the *slt*-IIvhb toxin operons are located on a 4.3 kb and a 4.9 kb *Pst*I fragment respectively (Ito *et al.*, 1990). The toxin operons from cosmids pSQ12 (*slt*-IIvha) and pJES54 (*slt*-IIvhb) were subcloned into high copy pBluescript vectors for further analysis (Table 3, see Figure 20 for restriction map and subclones of pJES54).

The three putative H414-36/89 toxin operons were cloned from isolated *Eco*RI fragments by PCR amplification as described in Materials and Methods. Supernatants from DH5α

Figure 18. 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*-IIvha probe by *in situ* gel hybridization. Numbers to the left are marker DNA fragments in kilobase pairs.

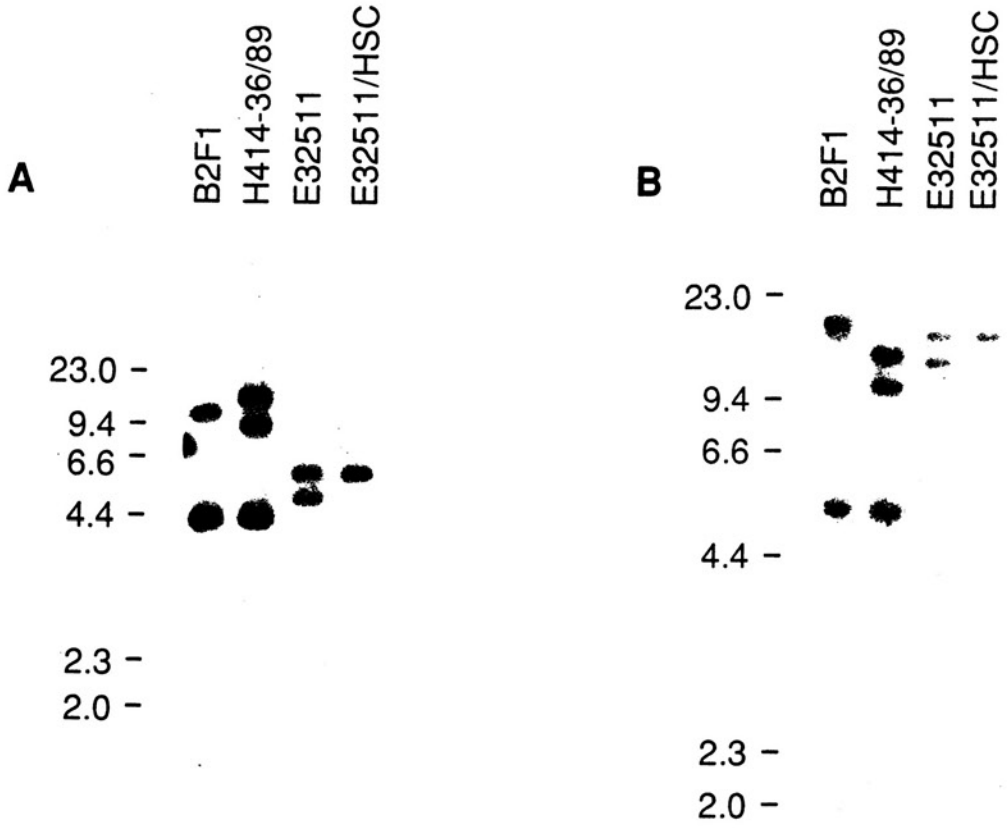

A
B2F1
H414-36/89
E32511
E32511/HSC
23.0
9.4
6.6
4.4
2.3
2.0
B
B2F1
H414-36/89
E32511
E32511/HSC
23.0
9.4
6.6
4.4
2.3
2.0

Figure 19. Identification of the *slt*-IIvha and *slt*-IIvhb genes isolated from a B2F1 cosmid bank. *In situ* gel hybridization of total DNA from strain B2F1 and plasmid DNA from cosmid clones pSQ12 (*slt*-IIvha) and pJES54 (*slt*-IIvhb) digested with *Pst*I and hybridized with an 841 bp *Sma*I-*Pst*I *slt*-II probe.

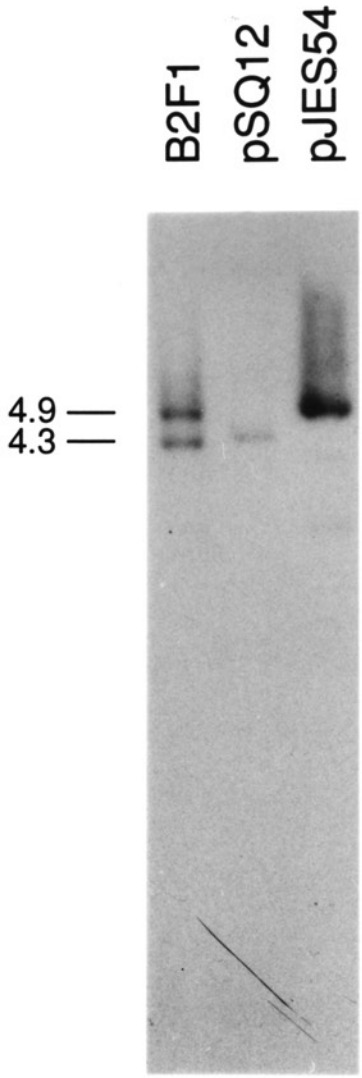
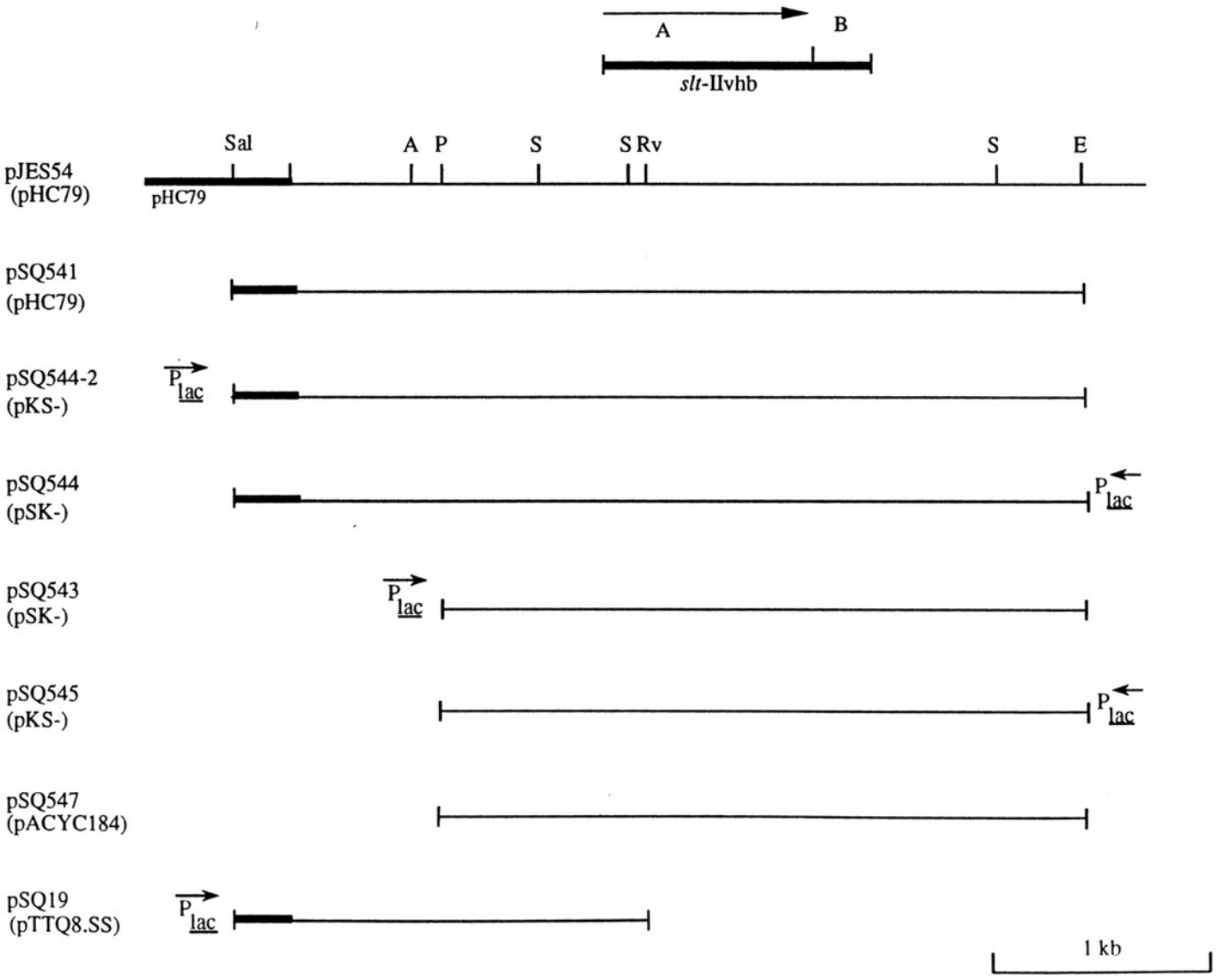

B2F1
pSQ12
pJES54
4.9
4.3

Figure 20. Construction of recombinant plasmids with the *slt*-IIvhb gene region. Fragments from the cosmid pJES54 were subcloned into the vectors indicated in parentheses. The location of the *slt*-IIvhb gene and the direction of transcription is indicated above the partial map of pJES54. The location of the P_{lac} promoters present in the pBluescript vectors are shown. Restriction sites: Sal, *Sal*I; A, *Acc*I; P, *Pst*I; S, *Sma*I; Rv, *Eco*RV; E, *Eco*RI.


A
B
slt-IIvhb
Sal
A
P
S
S Rv
S
E
pJES54
(pHC79)
pHC79
pSQ541
(pHC79)
pSQ544-2
(pKS-)
P lac
pSQ544
(pSK-)
P lac
pSQ543
(pSK-)
P lac
pSQ545
(pKS-)
P lac
pSQ547
(pACYC184)
pSQ19
(pTTQ8.SS)
P lac
1 kb

transformed with the resultant toxin clones, pSQ47, pSQ81, and pSQ135 were then subjected to cytotoxicity analyses along with *slt*-IIvha subclone pSQ343 and *slt*-IIvhb subclone pSQ543 (see below).

Cytotoxicity profiles of toxin preparations from B2F1 and H414-36/89 cloned toxin operons before and after incubation with anti-toxin antisera. Neutralization of SLT cytotoxicity is frequently used by investigators to classify SLTs (Scotland *et al.*, 1985; Strockbine *et al.*, 1986; Bockemuhl *et al.*, 1992). Toxin preparations from DH5α transformed with plasmids that expressed the various B2F1 and H414-36/89 toxin genes were titrated against standardized amounts of various antisera, and the CD_{50}/Antigen unit ratios of the toxin preparations were determined (Table 7). The extracts tested were prepared from DH5α containing pSQ343 (encoding SLT-IIvha), pSQ543 (encoding SLT-IIvhb), pSQ47 (from H414-36/89), and pSQ135 (from H414-36/89). The CD_{50}/Antigen unit ratios were low for the toxins encoded by pSQ543, pSQ343, pSQ47, and pSQ135, but the ratios were much higher for the toxins encoded by pJES120 (encoding SLT-II) or pSQ81 (from H414-36/89). The absolute values for the ratios depended on the specificity of the antiserum and the arbitrarily chosen standardized dose for that antiserum. Incubation of each of the toxin preparations with anti-SLT-I antisera had no effect on cytotoxicity (data not shown). Based on these patterns,

Footnotes for Table 7

[a] AJ65, 1:50 dilution.

[b] BC5 ascites, 1:100 dilution.

[c] 2E1 culture supernatant, undiluted.

[d] Ag units/ml, CD_{50}/ml toxin antigen that remains after incubation with antibody.

[e] CD_{50}/Ag unit, CD_{50}/ml of a toxin preparation divided by CD_{50} of toxin antigen units/ml of the homologous toxin preparation. Units given are arbitrary. Absolute values vary depending on the antibody used and cannot be compared between heterologous antibodies.

Table 7. Specific toxicity of SLT-II toxin preparations: ratio of cytotoxicity to immunoreactivity with various antisera raised against the prototypic SLT-II toxin.

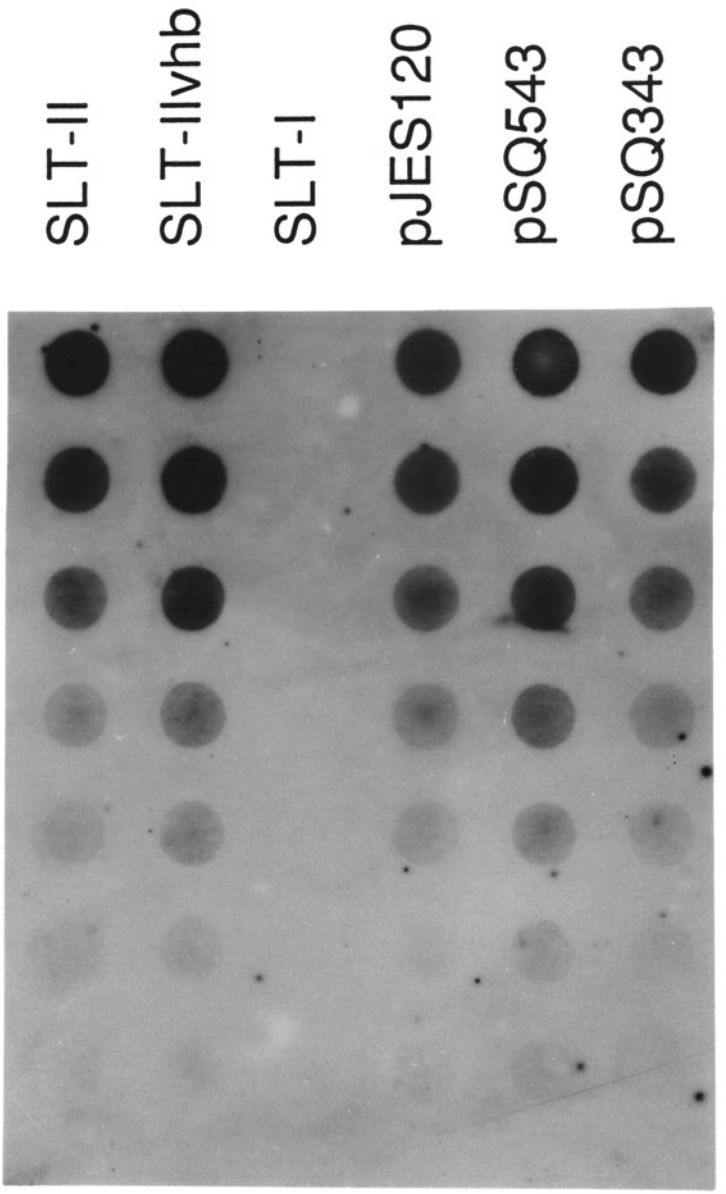
Toxin clone	Toxicity (CD_{50}/ml)	Anti-SLT-II[a]		Anti-SLT-II B[b]		Anti-SLT-II A[c]	
		Ag units/ml[d]	CD_{50}/ Ag unit[e]	Ag units/ml	CD_{50}/ Ag unit[e]	Ag units/ml	CD_{50}/ Ag unit[e]
pJES120	1×10^7	2.6×10^3	3,800	1.6×10^2	63,000	3×10^4	333
pSQ543	3×10^5	1×10^4	30	2.6×10^3	115	1.2×10^5	3
pSQ343	1×10^4	2×10^3	5	3.2×10^2	31	5.2×10^3	2
pSQ47	1.6×10^5	1×10^4	16	5.2×10^3	31	1.6×10^5	1
pSQ81	4×10^6	1×10^4	400	8×10^1	50,000	6×10^4	67
pSQ135	4×10^4	2.6×10^3	15	2.6×10^3	15	1×10^4	4
ϕH414-Lysogen	6×10^5	ND	ND	4×10^1	15,000	ND	ND

the H414-36/89 operons cloned into pSQ47 and pSQ135 encode SLT-IIc toxins (similar to SLT-IIvha and SLT-IIvhb), and the H414-36/89 operon present in pSQ81 encodes a toxin similar to the SLT-II prototype.

Immunodot blot analysis using a monoclonal antibody specific for the A subunit of SLT-II was done to quantify further the amount of toxin protein present in each preparation. Similar immunoreactivity (within a 2-fold difference) was observed between purified SLT-II and purified SLT-IIvhb, which suggests that the two toxins react equally well to this monoclonal antibody (Fig. 21). In addition, crude toxin preparations from DH5α that expressed SLT-II, SLT-IIvha, or SLT-IIvhb had comparable immunoreactivity within a 2-fold range (Fig. 21). Thus, a similar amount of toxin antigen was produced by DH5α transformed with each of the *slt* clones. These data provide strong support for the hypothesis that the SLT-II-related toxins are less toxic for Vero cells per unit of toxin protein than is the prototypic SLT-II toxin.

Bacteriophage induction and isolation. EHEC toxins *slt*-I and *slt*-II have frequently been isolated from toxin-converting lysogenic phage (O'Brien *et al.*, 1984; O'Brien *et al.*, 1989; Scotland *et al.*, 1983; Strockbine *et al.*, 1986; Willshaw *et al.*, 1987). For example, SLT-I toxin-converting phage H-19J was isolated from O26:H11 strain H-19, and SLT-II toxin-converting phage 933W was isolated from O157:H7 strain 933.

Figure 21. Immunoblot of crude toxin preparations for quantitation of toxin antigen. Serial two-fold dilutions of 100 ng of purified SLT-II and SLT-IIvhb, and 100 μl of a 10-fold dilution of sonic lysates from DH5α/pJES120 (SLT-II), DH5α/pSQ543 (SLT-IIvhb), or DH5α/pSQ343 (SLT-IIvha) were spotted onto nitrocellulose and hybridized with anti-SLT-II A monoclonal antibody. The intensities of the dot-blot color reactions were estimated visually.

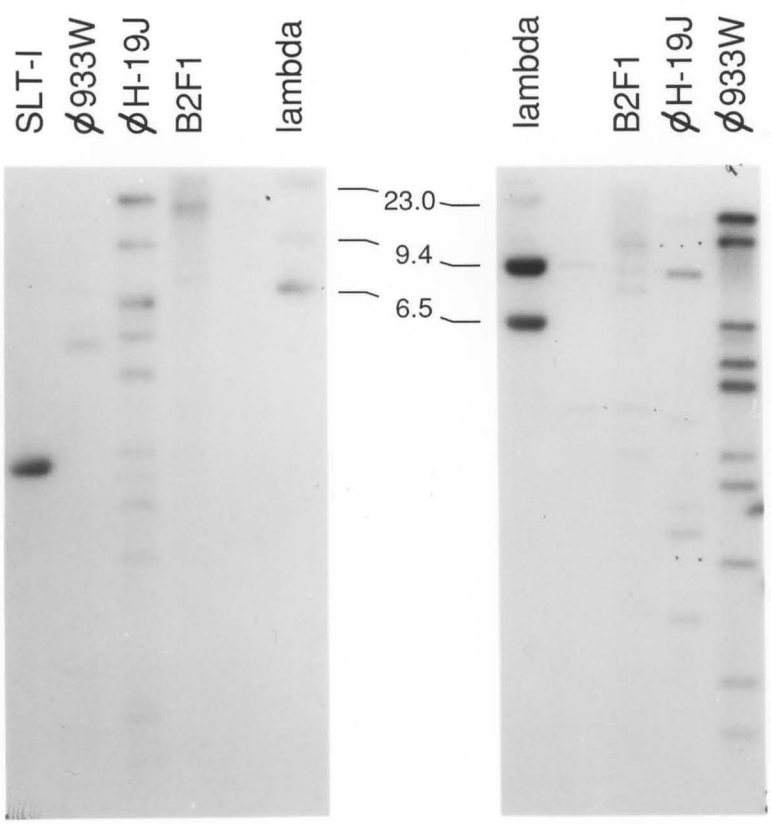

SLT-II
SLT-IIvhb
SLT-I
pJES120
pSQ543
pSQ343

To identify whether the toxin operons present in EHEC strains B2F1 and H414-36/89 were also located on lysogenic phage, several methods were employed to isolate phage from these strains. Phage was successfully isolated from strain H414-36/89. This phage was designated ϕH414. The cytotoxicity and CD_{50}/Antigen ratio of culture supernatants from three separate C600 lysogens derived from phage lysates of strain H414 indicated that ϕH414 encodes the H414-36/89 *slt*-II gene (Table 7 and data not shown).

Phage could not be isolated from strain B2F1. However, Southern blot analysis of DNA from this strain indicated that phage sequences were present (Fig. 22). Total DNA from ϕH-19J, ϕ933W, and B2F1 was digested with *Eco*RI and hybridized with either *Eco*RI-digested, nick-translated total DNA from ϕH-19J (Fig. 22, panel A) or ϕ933W (Fig. 22, panel B). A few B2F1 *Eco*RI fragments hybridized with ϕH-19J DNA and ϕ933W DNA. However, ϕH-19J and ϕ933W had numerous fragments that hybridized with the homologous as well as heterologous phage DNA probes. Taken together with the phage induction experiments, these results indicate that there are some SLT-toxin-converting phage sequences present in strain B2F1 but that such a phage may be defective.

Production of monoclonal antibodies to SLT-IIvhb. No monoclonal antibodies specific for SLT-II variant toxins have been described. Attempts were made during the course of this

Figure 22. Presence of SLT toxin-converting phage DNA sequences in EHEC strain B2F1. Total DNA was digested with *Eco*RI and hybridized with radioactively labeled (A) total DNA from ϕH-19J (*slt*-I) or (B) total DNA from ϕ933W (*slt*-II) by Southern blot hybridization. Numbers indicate length in kilobase pairs of HindIII digested lambda DNA fragments.


SLT-I
ϕ933W
ϕH-19J
B2F1
lambda
23.0
9.4
6.5
lambda
B2F1
ϕH-19J
ϕ933W
ϕH-19J
ϕ933W

study to isolate such a monoclonal antibody. Culture supernatants from 1,440 wells that contained hybridomas were screened for the capacity to neutralize SLT-IIvhb cytotoxicity. From the initial screen, three putative neutralizing hybridomas were isolated. One hybridoma neutralized 50 Vero CD_{50} of SLT-IIvhb at a 1:500 dilution. Unfortunately, the neutralizing activity of this particular hybridoma was reduced to a 1:64 titer after the hybridoma was subjected to limiting dilution for single cell cloning. This hybridoma and two other putative toxin-neutralizing hybridomas were frozen at -70°C. No further efforts to obtain an SLT-IIvhb monoclonal were made.

Regulation of *slt*-IIvhb toxin operon expression. Environmental signals such as temperature, iron, and osmolarity are known to control the expression of numerous bacterial virulence genes (Mekalanos, 1992). Weinstein *et al.* (1988a) demonstrated that *stx*/*slt*-I expression is regulated by iron and temperature when present in *S. dysenteriae*. As a first step to determine whether and how SLT-II-related toxin expression is regulated, an *slt*-IIvhb A subunit-*pho*A reporter gene fusion was constructed as described in Material and Methods (see Fig. 3). The production of the fusion protein by DH5α transformed with pSQ448 was visualized by Western blot analysis with both rabbit polyclonal anti-SLT-IIvh antibody and rabbit polyclonal anti-alkaline phosphatase antibody

(Fig. 23). A shift in molecular weight of the immunoreactive SLT-IIvhb A subunit from the expected 33,000 MW to a protein of approximately 75,000 MW is seen in panel A, Figure 23. A 75,000 MW (approx.) protein was also detected by anti-alkaline phosphatase antibody (Fig. 23, panel B). These results demonstrate that a toxin-alkaline phosphatase protein fusion was produced by the construct.

The construct was then used to measure temperature effects on toxin expression. The *slt*-IIvhb reporter gene fusion (located on pSQ448) was transformed into strain B2F1, and the level of fusion protein expressed was measured at 30°C and 37°C. Preliminary temperature regulation studies indicated that at most two-fold more fusion protein was generated when B2F1/pSQ448 was grown at 37°C than at 30°C (Table 8, experiment #1 and experiment #2). Because of these results and our unsuccessful attempts to replace the wild-type toxin gene in strain B2F1 with the reporter gene fusion by homologous recombination, we discontinued the temperature regulation studies.

In the process of designing a system for the analysis of toxin gene regulation in strain B2F1, DNA fragments containing the *slt*-IIvhb toxin operon were subcloned from pSQ541 and pJES54 to create pSQ544-2 and pSQ543, respectively. A 1,000-fold increase in Vero cell cytotoxicity was noted when a region upstream of the toxin operon was removed (compare

Figure 23. Western blot analysis of the SLT-IIvhb A-alkaline phosphatase protein fusion. Cellular lysates obtained by sonication of B2F1 and DH5α/pSQ448 were subjected to SDS-PAGE analysis as described in Materials and Methods. Protein from the SDS-PAGE was then transferred by Western blot onto nitrocellulose and hybridized with either polyclonal anti-SLT-IIvh (A) or polyclonal anti-alkaline phosphatase (B) antisera. The SLT-IIvhb A-alkaline phosphatase fusion protein migrated to approximately 75-80 kD as indicated by the arrow. The SLT-IIvhb A subunit present in the B2F1 lysate should migrate to approximately 33 kD but was not observed in this figure because the level of toxin produced by strain B2F1 was insufficient for detection by Western blot. The 33 kD band was evident in a subsequent experiment with toxin clone DH5α/pSQ543 and after SDS-PAGE of purified toxin preparations (data not shown and Figure 26).

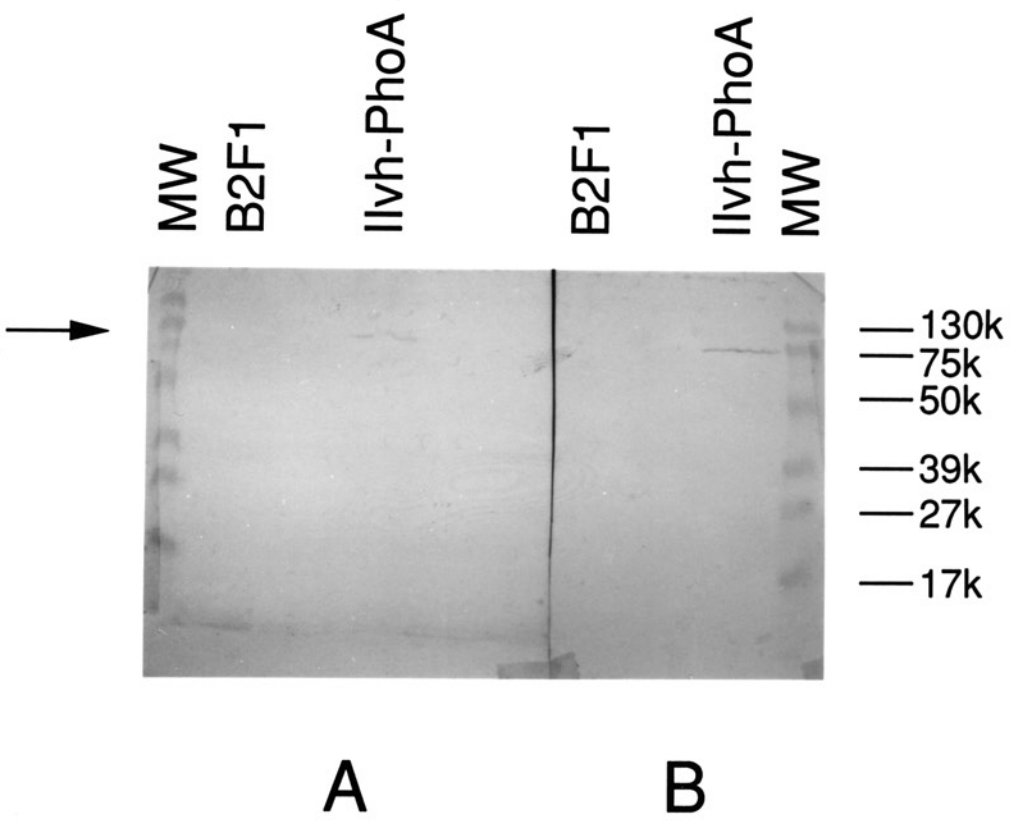

MW
B2F1
IIvh-PhoA
B2F1
IIvh-PhoA
MW
130k
75k
50k
39k
27k
17k
A
B

Table 8. Effect of temperature on *slt*-IIvhb A-*phoA* expression.

Construct	Temperature	OD_{600} of culture[a]	Alk. phosphatase activity[b]
Experiment #1			
	37°C	.986 ± .052	28.8 ± 0.8
B2F1/pSQ448	30°C	.650 ± .018	16.5 ± 0.5
	Ratio 37°C/30°C	1.5 : 1	2 : 1
B2F1	37°C	.536	1.7
Experiment #2			
	37°C	3.91 ± .09	10.0 ± 1.0
B2F1/pSQ448	30°C	4.34 ± .13	4.73 ± 1.3
	Ratio 37°C/30°C	1 : 1	2:1

[a] Arithmetic mean of four to five cultures ± 2 SEM (except B2F1 which was from one culture). Cultures were grown for 15 h in expt.#1 and 22 h in expt.#2.

[b] Units per milliliter culture. Arithmetic mean ± 2 SEM. Because of the variability of alkaline phosphatase units observed from experiment to experiment, data were not pooled. Two representative experiments are presented here.

levels produced by the larger subclone pSQ544-2 to those of the smaller subclone pSQ543) (Table 9). One possible explanation for this observation is that a negative regulator is present 5' to *slt*-IIvhb in pSQ544-2. Because part of the difference in cytotoxic levels between DH5α/pSQ543 and DH5α/pSQ544-2 may have been due to vector promotion of the smaller clone, the 2.8 kb toxin containing fragment in pSQ543 was cloned in the opposite orientation of P_{lac} (construct pSQ545). Only a 100-fold difference in cytotoxicity between DH5α expressing toxin from the cloned 2.8 kb fragment and DH5α expressing toxin from the 4 kb fragment was observed (Table 9). To assess whether the region upstream of *slt*-IIvhb could regulate toxin gene expression in trans, a 1.9 kb *Sal*I to *Eco*RV region from pSQ544-2 was cloned into pKS(-) (designated pSQ19-see Figs. 20 and 24) and co-transformed with pSQ547 (*slt*-IIvhb present on a compatible, lower-copy vector) into competent DH5α (designated DH5α/pSQ547/pSQ19) (Fig. 24). A 50-fold decrease in Vero cell cytotoxicity was observed between DH5α/pSQ547/pSQ19 and DH5α/pSQ547/pKS(-) in repeated assays (Tables 9 and 10). Unfortunately, further analysis of the putative regulatory region indicated that clone pSQ19 separately transformed into DH5α was unstable. Further attempts to re-isolate the putative regulator from either pSQ544-2 or pJES54 were uniformly unsuccessful. To date, the original strain DH5α/pSQ547/pSQ19 still produces significantly

Footnotes for Table 9

[a] The concentration of protein and OD_{600} of each culture was assessed and found to be approximately the same for all samples tested (5.5 - 6 mg protein/ml culture, OD_{600} 2.00).

[b] Cytotoxicity was below the level of detection.

Table 9. Level of SLT-IIvhb Vero cell cytotoxicity in the presence of putative regulatory region, experiment #1.

Toxin clone (fragment size, direction of P_{lac} promoter)	Vero cell cytotoxicity (CD_{50}/ml)[a]	Ratio of cytotoxicity
pSQ543 (P_{lac} → 2.8 kb)	5 x 10^5	pSQ543 : pSQ544-2 1,000 : 1
pSQ544-2 (P_{lac} → 4.0 kb)	5 x 10^2	pSQ545 : pSQ544-2 100 : 1
pSQ545 (P_{lac} ← 2.8 kb)	5 x 10^4	pSQ543 : pSQ545 10 : 1
pSQ547/pKS(-)	3.1 x 10^3	pSQ547/pKS(-) : pSQ547/pSQ19 48 : 1
pSQ547/pSQ19	6.4 x 10^1	-
pSQ19 (P_{lac} →)	< 4[b]	-

Figure 24. Schematic diagram of constructs used for analysis of the *slt*-IIvhb putative regulatory region. A 1.9 kb region was isolated from *slt*-IIvhb clone pSQ544-2 and ligated into pKS(-) to create pSQ19. Plasmid pSQ19 was then co-transformed into *E. coli* DH5α with *slt*-IIvhb on a compatible plasmid (pSQ547). The effect of the 1.9 kb region on toxin production was then measured by assessing the Vero cytotoxicity of DH5α/pSQ547/pSQ19 versus DH5α/pSQ547/pKS(-). The location of the *slt*-IIvhb operon (A and B subunit genes) is indicated by the internal arrow in both pSQ544-2 and pSQ547. The direction of promotion by P_{lac} in the pKS(-) clones is also noted.

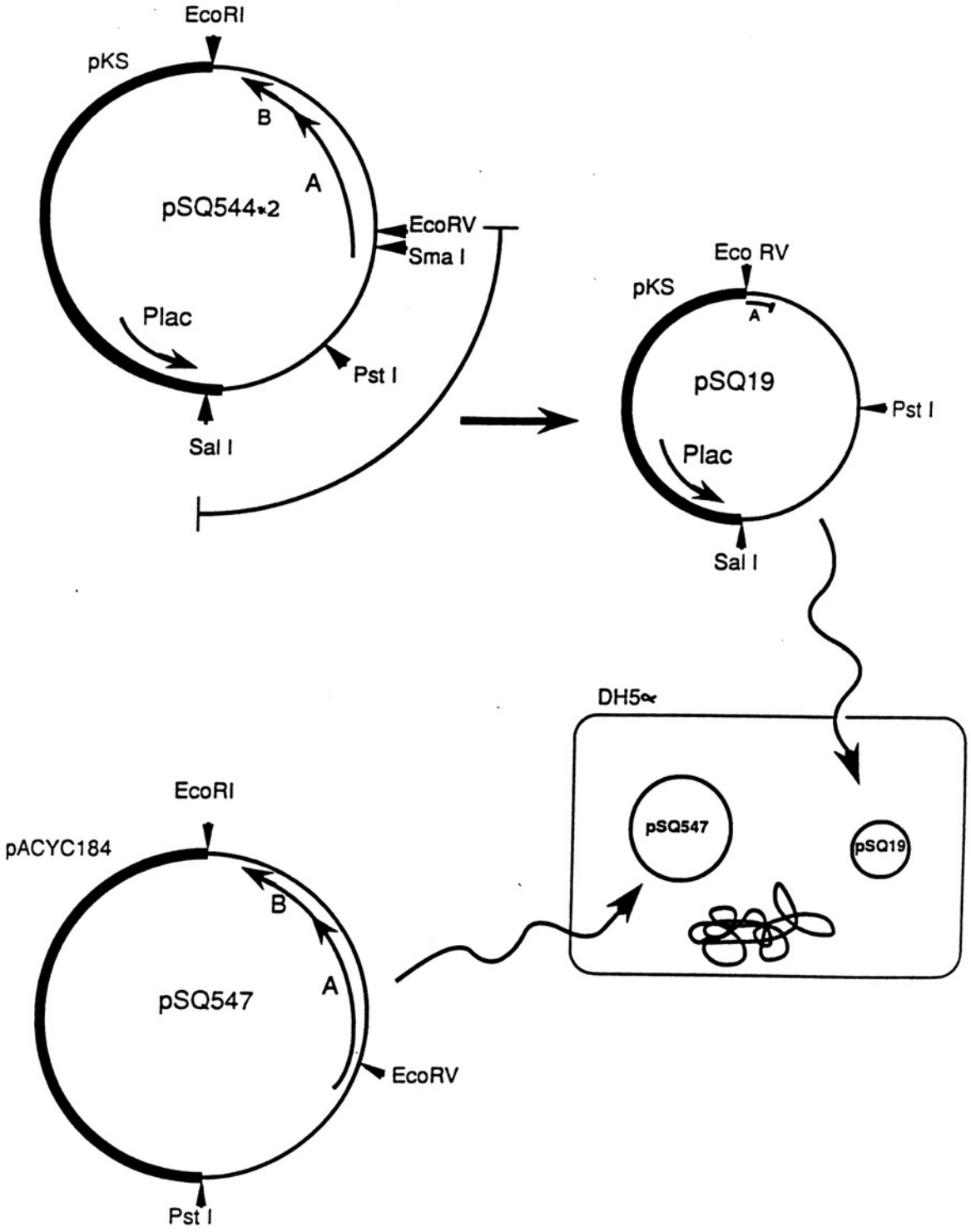

EcoRI
pKS
B
A
pSQ544*2
EcoRV
Sma I
Plac
Pst I
Sal I
Eco RV
pKS
A
pSQ19
Pst I
Plac
Sal I
DH5∝
pSQ547
pSQ19
EcoRI
pACYC184
B
A
pSQ547
EcoRV
Pst I

Table 10. Level of SLT-IIvhb Vero cell cytotoxicity in the presence of putative regulatory region, experiments #2 & #3.

Toxin clone	Log_{10} Vero CD_{50}/ml[a]	Ratio of cytotoxicity pSQ547/pKS(-) : pSQ547/pSQ19
Experiment #2		
pSQ547/pKS(-)	2.94 ± 0.29	25 : 1
pSQ547/pSQ19	1.54 ± 0.08	-
pSQ19	0.40 ± 0.17	-
Experiment #3		
pSQ547/pKS(-)	3.12 ± 0.15	41 : 1
pSQ547/pSQ19	1.5 ± 0	-

[a] Cytotoxicity values represent the geometric mean ± 2 SEM of five separate cultures. The concentration of protein in each culture in experiment #2 was assessed and found to be from 6 to 7 mg/ml in all samples tested. The cultures in experiment #3 were normalized for protein concentration prior to cytotoxicity testing.

lower levels of cytotoxicity than the control strain DH5α/pSQ547/pKS.

Oral inoculation of mice with *E. coli* DH5α that produces SLT-IIvha or SLT-IIvhb. Results from the mouse passive immunization studies indicated that production of SLT-II-related toxins was critical for the virulence of strain B2F1 in mice. To determine whether production of these toxins by an *E. coli* K-12 strain was sufficient for lethality of orally-infected mice, plasmids containing *slt*-IIvha or *slt*-IIvhb from strain B2F1, or *slt*-II (as a control) were separately transformed into DH5α(Str^r) and fed to streptomycin-treated mice. Production of either SLT-IIvha or SLT-IIvhb by DH5α(Str^r) was lethal for orally-inoculated mice (Table 11). Moreover, mice fed DH5α(Str^r) that elaborated SLT-IIvha or SLT-IIvhb developed lesions similar to those exhibited in mice fed B2F1(Str^r) (Fig. 25). These data support the hypothesis that the elaboration of SLT by EHEC is sufficient to cause death of orally-inoculated mice.

Previous studies by Wadolkowski *et al.* (1990b) indicated that DH5α(Str^r) that contained cloned *slt*-II toxin operons must produce high levels (>10^5 Vero cell cytotoxicity) of Shiga-like toxin to be lethal in the orally-inoculated mice. To assess the relative oral toxicity of SLT-IIvha and SLT-IIvhb in mice, DH5α(Str^r) strains that produced lower levels of these toxins, as determined by level of Vero cell

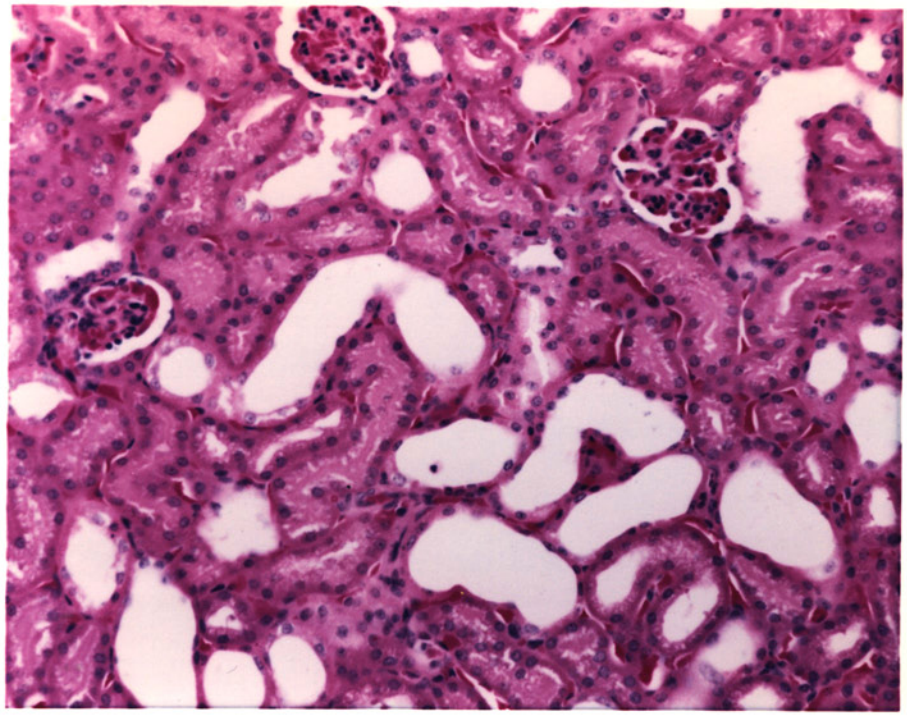
Table 11. Mouse virulence of *E. coli* DH5α(Str^r) expressing Shiga-like toxins

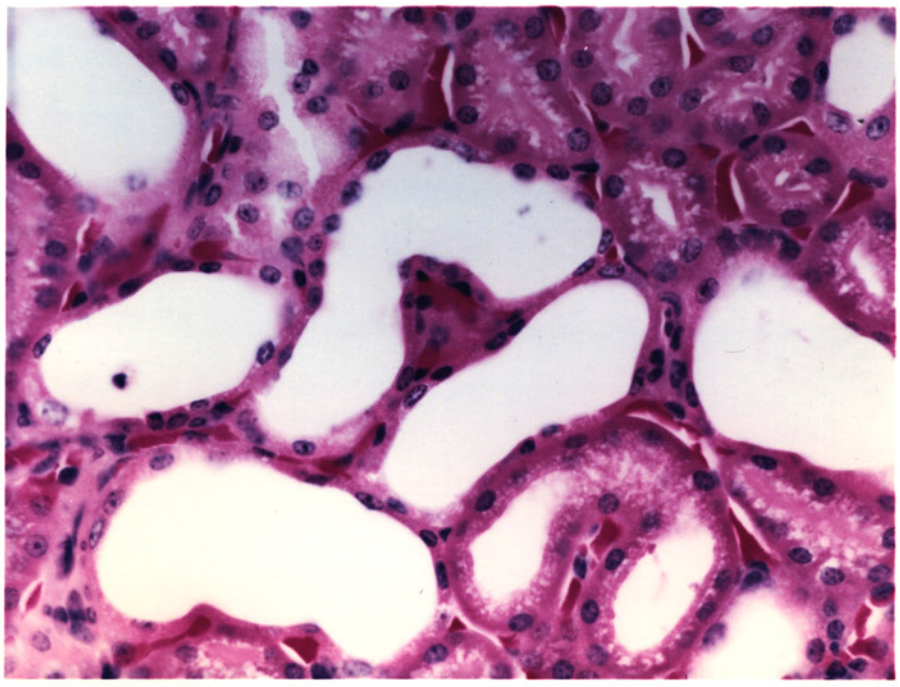
Plasmid in *E. coli* DH5α(Str^r)	SLT Toxin	Vero CD_{50}/ml culture[a]	Mouse death[b] (# dead/# fed)
pSQ343	IIvha	1 X 10^4	10/10
pSQ12	IIvha	5 X 10^2	10/10
pSQ543	IIvhb	1 x 10^6	23/23
pSQ544	IIvhb	1 x 10^4	14/14
pSQ545	IIvhb	5 x 10^4	9/9
pSQ549	IIvhb	1 x 10^2	0/10
pJES120	II	1 x 10^7	5/5
pLP32	II	1 x 10^6	5/5
pMJ100	II	1 x 10^5	1/20
pKS(-)	-	< 1 x 10^2	0/5
pBR328	-	< 1 x 10^2	0/5

[a] Cytotoxicity values were determined on the cultures that were fed to the mice. The lowest level of detection for this assay was 1 x 10^2 CD_{50}/ml.

[b] Mice were fed approximately 10^{10} CFU bacteria and monitored for death over 21 days. Each ratio represents pooled data from one to six experiments.

Figure 25. Photomicrograph of renal cortex from a streptomycin-treated mouse inoculated with 10^{10} CFU DH5α(Str^r)/pSQ543. The moribund mouse was sacrificed on day three post-inoculation, and the tissue was prepared as described in Materials and Methods. Note the dilated tubules with necrotic epithelial cells within the lumens of these tubules. Glomeruli appear histologically normal. Hematoxylin and eosin; magnification, X200 (top), X400 (bottom).





cytotoxicity, were fed to streptomycin-treated mice. These toxin-producing DH5α(Str^r) strains were created by cloning the *slt*-IIvha and *slt*-IIvhb operons into lower copy expression vectors (Table 3, Fig. 20). Results from this set of feeding experiments demonstrated that DH5α(Str^r) strains that produced SLT-IIvha at Vero cell cytotoxicity titers of 5 X 10^2 Vero CD_{50}/ml remained lethal in mice (Table 11). By contrast, DH5α(Str^r) that made SLT-II was only consistently lethal when the Vero CD_{50}/ml levels were ≥ 10^6 (Table 11). SLT-IIvhb which is 99% homologous to SLT-IIvha (Ito *et al.*, 1990) was not lethal at Vero CD_{50}/ml titers of 1 X 10^2. These data suggest that the level of SLT-IIvha/SLT-IIvhb production by *E. coli* required for lethality after oral infection is probably between 1 X 10^2 and 5 X 10^2 Vero CD_{50}/ml.

Comparison of *in vitro* and *in vivo* toxicity of purified SLT-II and SLT-IIvhb toxin. At least two possibilities can be proposed to explain the observation that *E. coli* DH5α(Str^r) transformants that produce ≥ 5 X 10^2 Vero CD_{50}/ml SLT-IIvha or SLT-IIvhb kill mice, whereas DH5α(Str^r) transformants that make as much as 10^5 Vero CD_{50}/ml SLT-II fail to do so: the specific activity of the toxins may differ 1) *in vitro* or 2) *in vivo*. To test these possibilities, SLT-II and SLT-IIvhb were purified, and the specific activities of the toxins were compared on Vero cells and in CD-1 mice.

A) Toxin purification. The yield and specific activity of SLT-II and SLT-IIvhb at each step in the purification are given in Table 12 and 13, respectively. Data shown in Table 13 are from the second of two SLT-IIvhb preparations. The SLT-II and SLT-IIvhb preparations appeared highly purified with only trace contaminants remaining as indicated by SDS-PAGE analysis (Fig 26). In the presence of β-mercaptoethanol, three bands of approximately 33,000, 28,000, and 10,000 MW were observed for SLT-IIvhb (Fig. 26). These bands correspond to toxin subunits A, A_1, and B, respectively (O'Brien *et al.*, 1983a; Yutsudo *et al.*, 1987). Western blot analysis of purified SLT-IIvhb indicated that the 33,000 and 28,000 MW bands seen in Figure 26 were toxin protein (Fig. 27). Only one band at 33,000 MW was seen on SDS-PAGE of purified SLT-II (Fig. 26). The absence of A_1 in the SLT-II preparation may indicate that this toxin was not cleaved by a protease during purification. Alternatively, the A_1 fragment may be present in the SLT-II preparation but at a concentration not detectable on this gel. Although the B subunit (10,000 MW) of SLT-IIvhb is observed on the Coomassie blue stained SDS-PAGE gel in Figure 27A, it is not immunoreactive with the polyclonal anti-SLT-IIvh antiserum and therefore is not detected by Western blot (Fig. 27B). The anti-SLT-IIvh antisera was prepared by Oku *et al.* (1989) from formaldehyde-treated, purified toxin from strain B2F1. In our hands, this antiserum does not neutralize SLT-IIvhb.

Table 12. Purification of SLT-II

Sample	Total protein (mg)	Sp. activity (CD_{50}/mg protein)	Recovery of cytotoxic activity (%)
Ammonium sulfate precipitate of clarified sonic lysate	1040	3.8×10^4	100
DEAE chromatography pooled fractions	150	2×10^6	75
Chromatofocusing chromatography pooled fractions	14.8	1×10^7	38
Affinity chromatography	0.002	2×10^8	0.01

Table 13. Purification of SLT-IIvhb

Sample	Total protein (mg)	Sp. activity (CD_{50}/mg protein)	Recovery of cytotoxic activity (%)
Ammonium sulfate precipitate of clarified sonic lysate	800	1.25×10^5	100
DEAE chromatography pooled fractions	45	7.4×10^5	33
Chromatofocusing chromatography pooled fractions	8.14	1.9×10^6	16
Affinity chromatography	0.017	2.1×10^6	0.04

Figure 26. Silver stained SDS-PAGE of purified SLT-IIvhb (0.17 μg) and SLT-II (0.02μg). SLT-IIvhb was purified from DH5α/pSQ543 twice. The numeral following the SLT-IIvhb toxin designation indicates the second purified SLT-IIvhb preparation. Numbers to the left of the gel indicate the molecular weights of marker proteins. Arrows on the left mark the A and A_1 subunits of the purified toxins. The B subunit is located at approximately 10,000 MW.

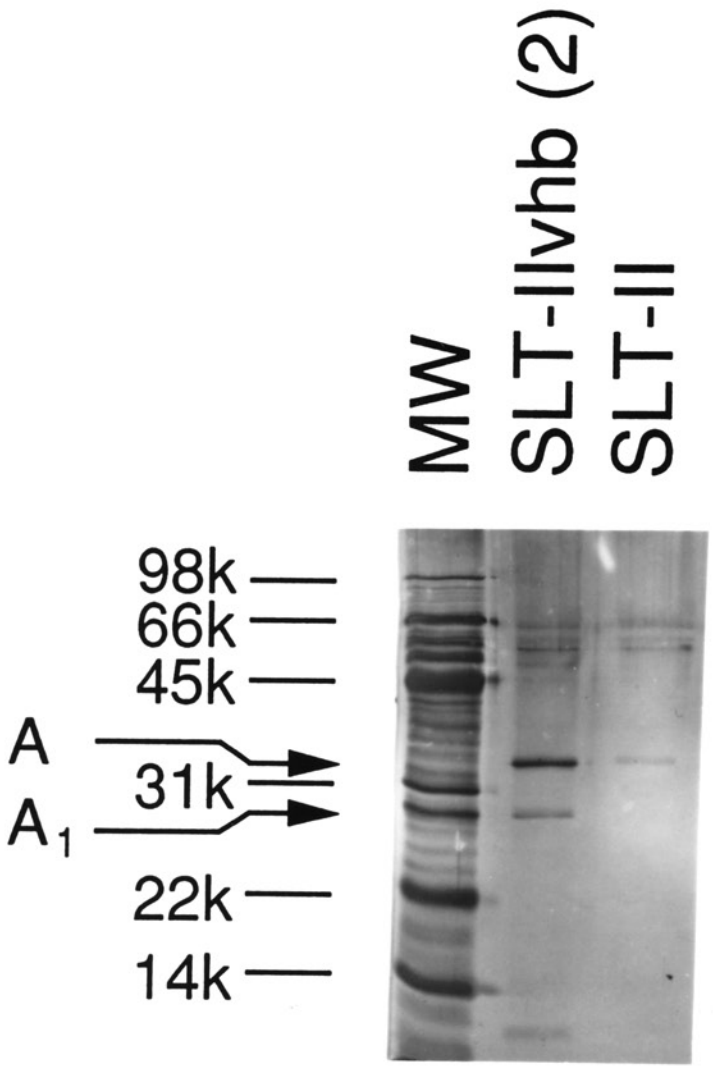
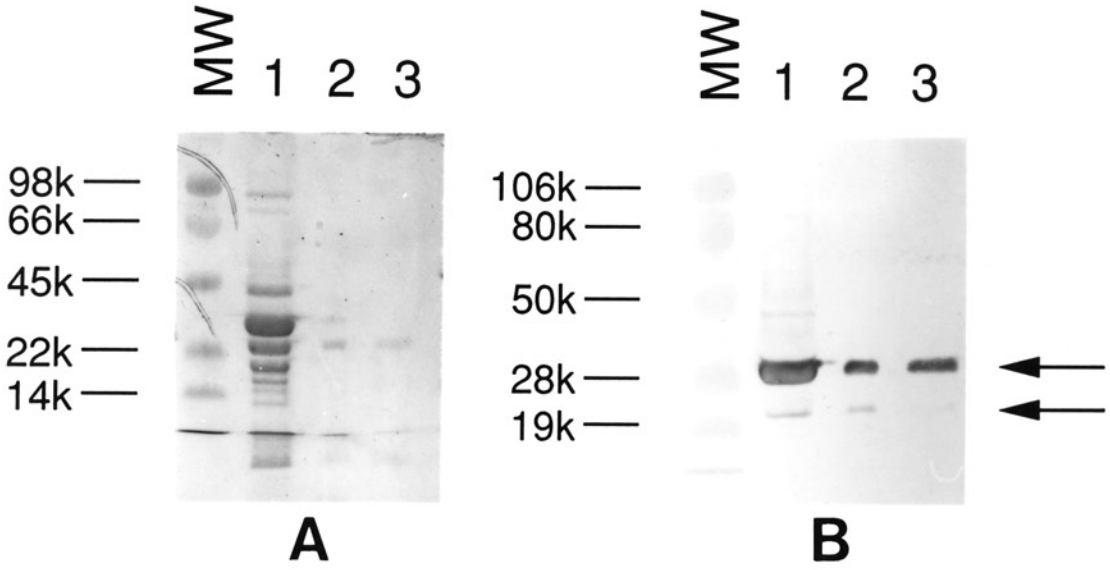

MW
SLT-IIvhb (2)
SLT-II
98k
66k
45k
A
31k
A1
22k
14k

Figure 27. SDS-PAGE and Western blot of purified SLT-IIvhb. SDS-PAGE (A) and Western blot (B) analysis of SLT-IIvhb before (Lane 1) and after (Lanes 2 and 3) affinity chromatography. The more homogeneous preparation in Lane 3 was dialyzed against PBS and used for subsequent toxin analysis. Numbers on the left of each panel indicate the molecular weights of marker proteins. Prestained markers (which are larger than the other marker proteins and may not represent the sizes given) were used for the Western blot. Arrows on the Western blot (to the right of panel B) mark the immunoreactive toxin A subunit (approximately 33,000 MW) and the nicked A_1 fragment of the A subunit (approximately 28,000 MW). Although the B subunit (10,000 MW) of SLT-IIvhb was observed on the SDS-PAGE gel (A), it was not immunoreactive with the polyclonal anti-SLT-IIvh antibody.


MW
1
2
3
98k
66k
45k
22k
14k
A
MW
1
2
3
106k
80k
50k
28k
19k
B

Because any B oligomers that had co-purified with holotoxin might interfere with toxin assays, the toxin preparations were analyzed by PAGE under non-denaturing conditions (Fig. 28). The hypothesis was that the holotoxin and B oligomers would migrate differently on these gels. However, only one band was observed for both SLT-II and SLT-IIvhb (Fig. 28). Moreover, Western blot analysis of the non-denaturing gel with polyclonal anti-SLT-II antibody, monoclonal anti-SLT-II A subunit antibody, or monoclonal anti-SLT-II B subunit antibody demonstrated that only one toxin band was present in the purified toxin preparations (Fig. 29) (Purified SLT-II B subunit was not available for use as a positive control). Thus, the B oligomers, if present, were at low concentrations in these toxin preparations, or our prediction that holotoxin and B oligomers would migrate differently was incorrect. Because SLT-II and SLT-IIvhb have approximately the same molecular weight, the differential migration of the holotoxins through the non-denaturing polyacrylamide gel was most likely due to the difference in the isoelectric point (pI) of the two molecules [pI of SLT-II = 4.1 (Yutsudo *et al.*, 1987); pI of SLT-IIvhb = 6.1 (Oku *et al.*, 1989)]. Purified SLT-I (pI = 7.0, gift from Dr. J. Samuel) also migrated differently from the other toxin proteins (Fig. 28).

Figure 28. Non-denaturing PAGE analysis of SLTs. Purified SLT-IIvhb (0.50 μg of preparation #1), SLT-IIvhb (0.17 μg of preparation #2), purified SLT-II (0.02 μg), and purified SLT-I (0.5 μg) (gift from Dr. J. Samuel) were subjected to non-denaturing PAGE and stained with coomassie brilliant blue as described in Materials and Methods. The SLT-II and SLT-I bands are faint in this photograph and are located in the upper portion of the gel.

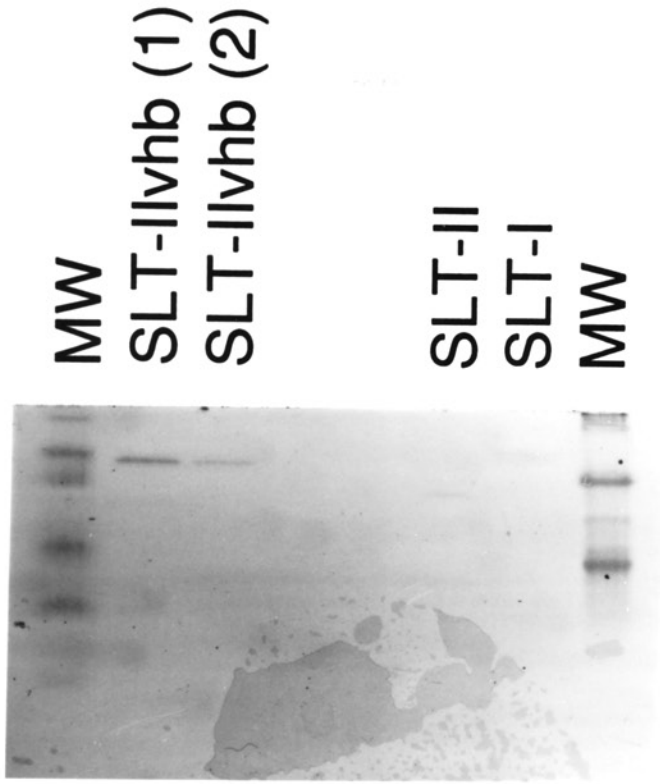
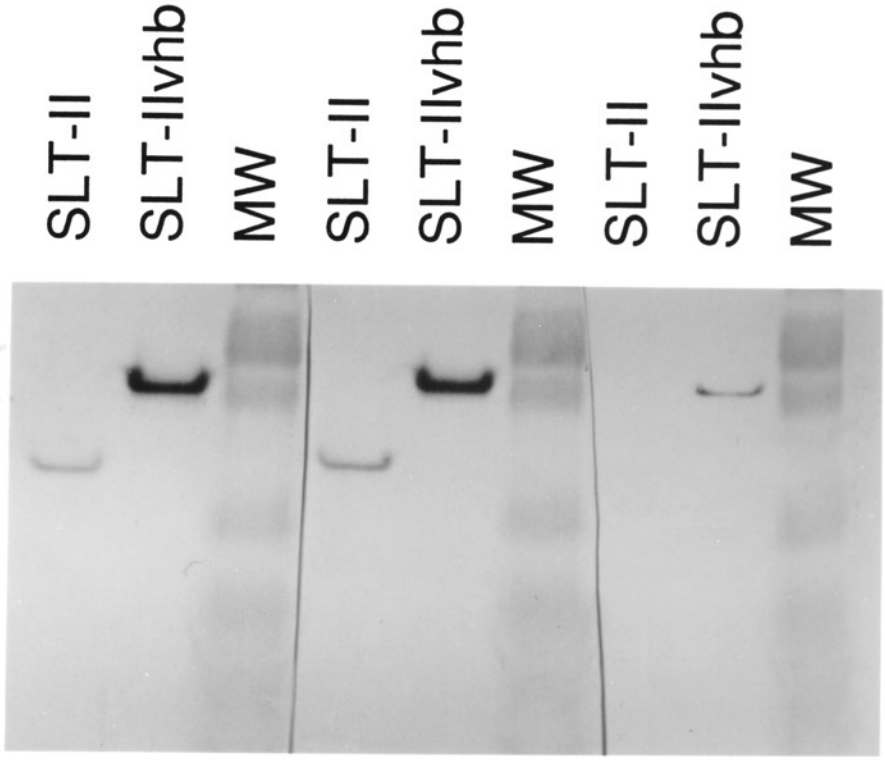

MW
SLT-IIvhb (1)
SLT-IIvhb (2)
SLT-II
SLT-I
MW

Figure 29. Western blot of purified SLT-II and SLT-IIvhb on non-denaturing PAGE. Purified preparations of SLT-II (0.02 μg) and SLT-IIvhb (0.17 μg) were separated by non-denaturing PAGE, transferred to nitrocellulose, and probed with polyclonal anti-SLT-IIvh antibody, monoclonal anti-SLT-II A antibody, or ascites from monoclonal anti-SLT-II B antibody as described in Materials and Methods. Prestained molecular weight markers were used to verify protein transfer and are in the far right lane of each blot. Because proteins migrate by both isoelectric point and size on non-denaturing PAGE, the fragment sizes of the marker proteins are not given.


SLT-II
SLT-IIvhb
MW
SLT-II
SLT-IIvhb
MW
SLT-II
SLT-IIvhb
MW
αSLT-II
αSLT-II A
αSLT-II B

B) Specific activity of purified SLT-IIvhb on Vero cells and in mice injected intraperitoneally. The specific activity of SLT-II on Vero cells was comparable to published results (Yutsudo *et al.*, 1987) and was approximately 5 pg/CD_{50} (2 X 10^8 CD_{50}/mg protein). However, the Vero cell specific activity of purified SLT-IIvhb was about 100-fold lower than SLT-II (approximately 500 pg/CD_{50} for both SLT-IIvhb toxin preparations). When the purified toxins were intraperitoneally injected into CD-1 mice, SLT-II and SLT-IIvhb were equally toxic. The specific activity of both SLT-II and SLT-IIvhb was 1-2 ng/LD_{50}. These results indicate that SLT-II is more toxic for Vero cells than SLT-IIvhb but that the toxins are equally active in mice. Therefore, the apparent difference in *in vivo* toxicity of DH5α(Str^r)-transformants that make SLT-IIvha or SLT-IIvhb compared to DH5α(Str^r)-transformants that produce SLT-II reflects the use of Vero CD_{50} as a measure of toxicity.

Analysis of SLT-IIvhb B subunit mutants. There are five amino acid differences in the A subunit between SLT-II and SLT-IIvhb and only two amino acid differences in the processed B or receptor-binding subunit (Ito *et al.*, 1990). To determine whether one or both of the amino acid differences in the B subunit of the SLT-II-related toxins were integral to the differential cytotoxicity of these molecules, Dr. J. Samuel created two single and one double amino acid substitution(s)

in SLT-IIvhb (from pSQ543). These mutants: N16D, A24D, and N16D/A24D correspond to the amino acid codons present in the B subunit of SLT-II at those positions. In this study, the cytotoxic titers (CD_{50}/ml culture) of DH5α transformed with *slt*-IIvhb or the B subunit mutants were found to be: pSQ543, 5 X 10^5; N16D, 1 X 10^7; A24D, 1 X 10^5; N16D/A24D, 1 X 10^6. The high level toxin-producing DH5α transformants grew poorly as indicated by the low cell density of overnight cultures. To circumvent problems that plasmid copy number might cause on cell growth, the *slt*-IIvhb operons containing the B subunit mutations were separately cloned into a lower copy vector (pBR328). Toxin preparations from the resultant lower-copy *slt*-IIvhb mutant toxin clones were subjected to further analysis. The cytotoxicity of toxin preparations containing N16D and N16D/A24D, as well as the CD_{50}/Antigen unit ratio of these mutant proteins, resembled SLT-II more than the parental SLT-IIvhb (Table 14). Taken together, these data indicate that the asparagine residue at position 16 is a critical determinant of the SLT-IIvhb toxin phenotype.

Footnotes for Table 14.

[a] AJ65, 1:50 dilution.

[b] BC5 ascites, 1:100 dilution.

[c] 2E1 culture supernatant, undiluted.

[d] Ag units/ml, CD_{50}/ml toxin antigen that remains after incubation with antibody.

[e] CD_{50}/Ag unit, CD_{50}/ml of a toxin preparation divided by CD_{50} of toxin antigen units/ml of the homologous toxin preparation. Units given are arbitrary. Absolute values vary depending on the antibody used and cannot be compared between heterologous antibodies.

Table 14. Specific toxicity of SLT-IIvhb B subunit mutant toxin preparations: ratio of cytotoxicity to immunoreactivity with various antisera raised against SLT-II.

Toxin clone	Toxicity (CD_{50}/ml)	Anti-SLT-II[a]		Anti-SLT-II B[b]		Anti-SLT-II A[c]	
		Ag units/ml[d]	CD_{50}/ Ag unit[e]	Ag units/ml	CD_{50}/ Ag unit[e]	Ag units/ml	CD_{50}/ Ag unit[e]
pJES120	1×10^7	2.6×10^3	3,800	1.6×10^2	63,000	3×10^4	333
pSQ543	1.3×10^6	2×10^4	65	3.2×10^5	4	4×10^4	33
N16D	1.6×10^5	1.6×10^2	1,000	4×10^1	4,000	1.3×10^3	123
A24D	5.2×10^3	6.4×10^2	8	1.3×10^3	4	6.4×10^2	8
N16D/A24D	1.6×10^5	3.2×10^2	500	1.6×10^2	1,000	1.6×10^2	1,000

DISCUSSION

I. Pathology of mice infected with 091:H21 EHEC strains.

In this study, several EHEC strains that produce SLT-II and/or SLT-II-related toxins were fed to streptomycin-treated mice. Two of the strains tested had an LD_{50} of <10 organisms: 091:H21 strain B2F1 (isolated from a Canadian patient with HUS) and 091:H21 strain H414-36/89 (isolated from a German patient with hemorrhagic colitis). Histopathological examination of kidneys from the moribund mice fed 091:H21 strains B2F1 and H414-36/89 revealed degeneration and necrosis of renal tubules. This pattern of kidney damage is consistent with that observed in mice fed EHEC strain 933cu-rev (Wadolkowski *et al.*, 1990a). 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 kidney (Tesh *et al.*, 1993).

Blood chemistry findings from B2F1-infected mice also deviated from typical laboratory results observed in human HUS. Patients with HUS have renal glomerular damage, thrombocytopenia, and hemolytic anemia (Levin *et al.*, 1989; Neill *et al.*, 1987; Ratnam *et al.*, 1985). The B2F1-infected mice had slight increases in hemoglobin, hematocrit, and platelet counts, findings which suggest hemoconcentration.

Because hemoconcentration occurs frequently in moribund animals (Dr. J. Burris, personal communication), this observation was not surprising. Furthermore, it has been hypothesized that the hemolytic anemia of HUS occurs when erythrocytes are ruptured as they pass through damaged renal glomeruli (Bull *et al.*, 1967; Habib *et al.*, 1981). 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 raised, the hemoconcentration effects obscure the interpretation of these renal function results. On the basis of the histopathology and hematology data, it can be concluded that the streptomycin-treated mouse model is not a model for HUS.

Even though EHEC-infected mice do not demonstrate all of the features associated with clinical HUS, the mouse model has proven to be a worthwhile model for EHEC infection. Unlike some other models for this disease, the route of infection in the mouse model and in humans is the same. In addition, the bacteria colonize the mouse intestine and are shed in the feces as is the case in human EHEC infection. Moreover, as has been suggested in the human disease, toxin production by EHEC appears to be critical for the virulence of the infecting bacterium in the orally-inoculated mouse. Taken together, these observations support the proposition that the

streptomycin-treated mouse model is a useful tool for assessing virulence of orally-administered EHEC. This model may also be valuable in the development of an anti-toxin vaccine.

II. Virulence assessment of O91:H21 strains B2F1 and H414-36/89.

The virulence of strains B2F1(Str^r) and H414-36/89(Str^r) was directly correlated with SLT-II and/or SLT-II-related toxin production by the 091:H21 EHEC isolates, 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 091:H21 isolates. Thus, the mere presence of *slt*-II or *slt*-IIc in an EHEC strain does not guarantee virulence for mice.

One possible explanation for the marked virulence of the 091:H21 isolates is that these EHEC strains are particularly efficient at systemic delivery of toxin from the gut. Because the site at which EHEC colonize the intestine of streptomycin-treated mice and the mechanism by which the bacteria adhere could affect delivery of toxin, several facets of EHEC 091:H21-intestinal interaction were evaluated. The two 091:H21 virulent strains were found to colonize the mouse small intestine, a region that may facilitate significant systemic absorption of Shiga-like toxin. However, colonization was not consistently demonstrated at this site.

The variable small bowel colonization data suggest that EHEC 091:H21 strains might grow to high numbers at that intestinal location but may not adhere avidly to the epithelial cell surface. Indeed, Dr. A. Melton (Dept. Microbiology, USUHS) found that the two 091:H21 strains grew readily in small intestinal mucus, whereas the non-lethal strain 933 did not. Though this growth characteristic may enhance the systemic delivery of toxin, it was not limited to the 091:H21 strains; strain E32511(Str^r) which killed only three out of twenty-three mice fed 10^{10} organisms also grew well in small intestinal mucus. These data suggest that although the capacity to grow in mucus isolated from the small intestine may be important for virulence, there must be another component(s) involved since E32511 was less virulent in orally-inoculated, streptomycin-treated mice than B2F1 and H414-36/89.

One difference between the 091:H21 strains and the other EHEC strains tested is that the 091:H21 strains do not contain the *eae* gene (M. McKee, unpublished data) which has been suggested to be involved in adherence of prototypic EHEC strains (reviewed by Tesh *et al.*, 1992). That the 091: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.

The renal histopathology and lethality observed in mice fed DH5α(Str^r)/*slt*-IIvha and DH5α(Str^r)/*slt*-IIvhb was further evidence that SLT-II-related toxin production is critical for the virulence of EHEC strains B2F1 and H414-36/89. The finding that purified SLT-IIvhb and SLT-II were equally toxic in intraperitoneally-injected mice suggests that mouse lethality is not dependent on the SLT-II subtype produced by an EHEC isolate. However, *slt*-IIvhb toxin gene expression may be up-regulated *in vivo*, as suggested by the *in vitro* gene regulation studies conducted during this project.

Although purified SLT-IIvhb was 500-fold less toxic than SLT-II on Vero cells, DH5α/*slt*-IIc that produced 1,000 to 5,000-fold less Vero CD_{50}/ml than DH5α/*slt*-II remained lethal in orally-inoculated mice. The observed discrepancy in Vero CD_{50} between death of mice inoculated with purified toxins and death of mice fed DH5α that contained cloned toxin operons could be due to an up-regulation of *slt*-IIvhb toxin gene expression *in vivo*. Alternatively, these Vero cell CD_{50} differences may reflect nothing more than normal variation in titration of CD_{50}/ml.

III. Host-immune response to EHEC and disease.

The passive immunization data described in this dissertation suggest that SLT-II/IIc causes renal tubular necrosis in the EHEC-infected animal. In support of this hypothesis, Tesh *et al.* (1993) recently demonstrated that the

functional Shiga-like toxin receptor Gb_3 is present on mouse renal tubular cells. In addition, these investigators demonstrated that SLT-I and SLT-II bind primarily to the tubular epithelial cells in frozen tissue sections of normal mouse renal cortex. However, no evidence of direct toxin binding to mouse kidney has been obtained after orally-inoculating mice with strain 933cu-rev (Wadolkowski, *et al.*, unpublished results). The inability to detect toxin on the mouse kidney cells may be solely a technical issue if the level of toxin that mediates the kidney damage is below the level of detection. Alternatively, the fact that SLT has not been isolated from the kidneys or blood of infected mice may suggest that the kidney damage observed is due in some way to the host-immune response to toxin production. It should be noted that SLT has also not been isolated from the blood of EHEC infected patients.

Results from experimental human and monkey trials using tox(-) *Shigella dysenteriae* strains suggest that toxin production by the bacteria causes damage to the colonic microvasculature (Levine *et al.* 1973; Fontaine *et al.*, 1988). This damage may in turn allow LPS from the infecting organism and/or from the normal flora to be released at greater levels systemically. The systemic introduction of elevated levels of endotoxin would result in a cascade of immunological events including the release of numerous cytokines.

One of the cytokines that is released by monocytes in response to LPS is tumor necrosis factor (TNF). Recent data suggests that TNF-α increases the level of Gb_3 on human endothelial cells *in vitro* (van de Kar et al. 1992). With this observation in mind, one model for the cascade of events leading to kidney damage after EHEC infection is as follows. The release of Shiga-like toxin in the intestine causes colonic microvascular damage which leads to the release of both SLT and LPS into the vasculature. LPS induces TNF production which causes a concurrent increase in Gb_3 molecules on renal tubular epithelial or glomerular endothelial cells. The increased levels of toxin receptors causes these cells to become sensitive to finite quantities of toxin released from the gut. In turn, the protein synthesis of the targeted cells is inhibited by the toxin. These kidney cells then die which results in renal damage and eventual kidney failure.

IV. Characterization of SLT-II-related toxins.

In situ hybridization experiments conducted during this study revealed that strain H414-36/89 contained three *slt*-II operons. Recently, several labs have isolated EHEC strains that carry two toxin operons from the *slt*-II group (Hii *et al.*, 1991; Tyler *et al.*, 1991; Schmitt *et al.*, 1991). To our knowledge, this is the first definitive report of a clinical isolate that contains three toxin operons from the SLT-II group. Two of the H414-36/89 toxins expressed from

operons cloned from this strain had low CD_{50}/Antigen unit ratios similar to the SLT-II-related toxins (SLT-IIc). The gel migration pattern of the 4.1 kb *Eco*RI fragment containing one of these operons and the *Eco*RI fragment containing the *slt*-IIvhb operon from B2F1 were indistinguishable. Similarly, the size of one of the *slt*-II-probe positive *Hind*III (Fig. 18), *Pst*I, or *Bam*HI (data not shown) fragments of H414-36/89 DNA was nearly identical to the *slt*-IIvhb fragment of B2F1. Although the nucleotide sequences of the *slt* operons from H414-36/89 were not determined, the cytotoxin immunoreactivity and hybridization results support the tenet that this strain carries one copy of *slt*-II and two copies of *slt*-IIc.

Analysis of the SLT-IIvhb B subunit mutants indicated that Asn-16 was responsible for the reduction in cytotoxicity and decreased CD_{50}/Antigen unit ratio of SLT-IIvhb on Vero cells when compared to SLT-II. As mentioned previously, both Shiga toxin mutagenesis (Jackson *et al.*, 1990b) and X-ray crystallographic results (Stein *et al.*, 1992) indicate that position 17 in the Shiga toxin/SLT-I B subunit (which corresponds to position 16 in SLT-IIvhb) is involved in toxin-receptor binding. The SLT-II-related toxins, SLT-IIvha, SLT-IIvhb, and SLT-IIc, contain an uncharged asparagine residue at position 16, whereas Shiga/SLT-I and SLT-II have a negatively charged aspartic acid residue at this location (see Fig. 1 for SLT-II toxin comparison). The lack of a negatively charged amino acid at position 16 in the SLT-II-related toxins

may affect the electrostatic interaction between the amino acids in the putative toxin-receptor binding cleft and result in a reduction in toxin-receptor binding. In support of this possibility, Samuel *et al.* (1990) recently demonstrated that SLT-IIvhb does not bind to the functional cell surface receptor (Gb_3) with as high an affinity as the prototypic SLT-II toxin. Dr. J. Samuel is currently comparing the parent and the SLT-IIvhb B subunit mutants for avidity of binding to Gb_3. Previous studies by Jackson *et al.* (1990b) revealed a 16% reduction in receptor analog binding by a Shiga/SLT-I-N17D mutation (corresponds to residue 16 in SLT-IIvhb).

There are also A subunit differences between SLT-IIvha/SLT-IIvhb and SLT-II that could account for the differential level of cytotoxicity observed between these toxins and SLT-II on Vero cells. Preliminary assays in which the enzymatic activity of purified SLT-IIvhb and SLT-II were compared indicated that there was no difference in enzymatic activity of these two toxins (S. Lindgren, data not shown). Moreover, SLT-IIc (from EHEC strain E32511) which is identical to SLT-II in the A subunit and SLT-IIvha in the B subunit (Fig. 1) has the same cytotoxicity and immunoreactivity profile as SLT-IIvha/SLT-IIvhb (Schmitt *et al.*, 1991). In addition, Dr. C. Schmitt found that mutant SLT-IIc/N16D is phenotypically the same as SLT-IIvhb/N16D (unpublished data). Thus, the only difference between SLT-IIvha/SLT-IIvhb and

SLT-II that is relevant to cytotoxicity appears to be the Asn-16 residue in the B subunit.

V. Evolutionary and diagnostic implication of O91:H21 EHEC virulence.

The serotype, level of virulence, *slt*-IIc/IIvhb *in situ* hybridization results, plasmid profile, and *eae*- phenotype of strains B2F1 and H414-36/89 suggest that these EHEC isolates may be clonally related. One difference between these two strains, however, is that strain H414-36/89 carries an SLT-II toxin-converting lysogenic phage. Conceivably, this phage could have originally been present in strain B2F1 but spontaneously lost upon passage through the patient, the moose, or at some earlier time. Spontaneous loss of SLT-II phage from EHEC isolates has been observed after human infection [strain 87-23 (Dr. P. Tarr, personal communication)] and after laboratory passage [strain E32511/HSC from E32511 (Hii *et al.*, 1991); 933D from strain 933 (O'Brien *et al.*, 1989)]. Techniques such as restriction fragment length polymorphism analysis of rDNA (Strockbine *et al.*, 1990), multilocus enzyme electrophoresis (Selander, *et al.*, 1986), and esterase electrophoretic polymorphism/specific activity (Goullet *et al.* 1989, 1990) could be used to more closely assess the overall genetic similarity of these two EHEC strains. Although such analyses were not part of this study, the clonal descent of pathogenic EHEC strains from particular

serogroups has previously been demonstrated (Mariani-Kurkdjian *et al.*, 1993; Whittam *et al.*, 1988). The fact that strain B2F1 and H414-36/89 were isolated from patients from two geographically distant countries makes the presumptive clonality of these 091:H21 strains particularly intriguing.

The striking virulence of strains B2F1 and H414-36/89 is 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 0157:H7 or 0157:H- EHEC serotype and the observation that many 0157:H7 and 0157:H- strains do not ferment sorbitol (March *et al.*, 1986). EHEC strains H414-36/89 and B2F1 are of the 091: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 (Bockemuhl *et al.*, 1992). To facilitate such identification, *slt* probes have been generated (Newland *et al.*, 1988; Karch *et al.*, 1989; Hii *et al.*, 1991; Tyler *et al.*, 1991; Gannon *et al.*, 1992) and procedures that identify *slt* from isolated colonies using PCR technology have been developed (Karch *et al.*, 1989; Pollard *et al.*, 1990;

Jackson *et al.*, 1991; Hii *et al.*, 1991; Tyler *et al.*, 1991; Mariani-Kurkdjian *et al.*, 1993). In addition, Gannon and colleagues recently developed a PCR method for the detection of *slt* in ground beef (Gannon *et al.*, 1992).

VI. Summary

In conclusion, two clinical EHEC isolates have been identified that are highly pathogenic for orally-infected, streptomycin-treated mice. The studies detailed here indicate that death of these mice is 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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