THE SHIGA AND SHIGA-LIKE CYTOTOXINS: GENE REGULATION AND FUNCTIONAL ANALYSIS OF THE BINDING SUBUNITS

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ABSTRACT

Title of Dissertation: The Shiga and Shiga-like Cytotoxins: Genetic Regulation and Functional Analysis of the Binding Subunit.

Debra Lynne Weinstein; Candidate, Doctor of Philosophy, 1989

Dissertation directed by: Alison D. O'Brien, Ph.D., Professor, Department of Microbiology

Strains of Escherichia coli produce cytotoxins which are related to the Shiga toxin produced by Shigella dysenteriae type 1. Shiga-like toxin type I (SLT-I) and Shiga-like toxin type II (SLT-II) are produced by enterohemorrhagic E. coli which cause hemorrhagic colitis and/or the hemolytic uremic syndrome in humans. Shiga toxin, SLT-I, and SLT-II are primarily cell-associated cytotoxins that kill both Vero cells and HeLa cells in culture. A third SLT, the Shiga-like toxin type II variant (SLT-IIv), is produced by strains of E. coli responsible for the edema disease of swine. SLT-IIv, which is antigenically related to SLT-II, is markedly more cytotoxic for Vero than HeLa cells. Structurally, all of these toxins are comprised of an A subunit, which is responsible for the enzymatic activity, and multiple B subunits which bind the toxin to a eucaryotic cell receptor.

The first major objective of this study was to examine the

genetic organization and regulation of the Shiga toxin and SLT operons. Mini-Mu lac operon fusions were isolated in the cloned slt-I genes and used to confirm the location of the slt-I A and slt-I B genes and the direction of the transcription, and to support previous evidence that there is a second promoter for the slt-I B gene distal to the slt-I A gene. This second promoter may enhance the transcription of the B subunit. A subsequent comparison of the Shiga toxin, SLT-I, SLT-II and SLT-IIv operons demonstrated that the organization of the operons of all the toxin family members is similar. In addition, each operon has a conserved ribosome binding sequence both 5' to the A subunit genes and in the untranslated space between the subunit genes, which may enhance the translation of the B subunit.

A second aspect of the regulation studies was to determine the influence of iron on SLT-I, SLT-II, and SLT-IIv production in E. coli. The effect of temperature on Shiga toxin and SLT-I production in S. dysenteriae type 1 and E. coli, respectively, was also analyzed. Iron is known to depress Shiga toxin production by S. dysenteriae 1, and temperature has been shown to regulate several genes required for Shigella invasiveness. SLT-I production in an E. coli lysogenized with a toxin-converting phage, like Shiga toxin production in S. dysenteriae type 1, was found to be suppressed by iron, whereas SLT-IIv and SLT-II production in E. coli were not affected by iron. Shiga toxin production in S. dysenteriae 1 strain 60R was found to be regulated by temperature, while temperature had no effect on SLT-I production in E. coli.

Taken together, these results indicate that the regulation of synthesis of SLT-IIv and SLT-II differs from that of Shiga toxin/SLT-I and that differences do exist (i.e. temperature) between regulation of Shiga toxin in S. dysenteriae and SLT-I in E. coli.

The second major objective of this project was to clone the structural genes for SLT-IIv from an ED-causing strain of E. coli The nucleotide and deduced amino acid sequences were determined and compared to the other members of the Shiga toxin family. The A subunit genes for SLT-IIv and SLT-II were highly homologous (94%), whereas the B subunit genes were less homologous (79%). The SLT-IIv genes were distantly related (55 to 60% overall homology) to the genes for Shiga toxin and SLT-I. As with Shiga toxin, SLT-I and SLT-II, the A subunit of SLT-IIv had regions of homology with the plant toxin ricin. One of these regions may contain the active site of the molecule.

The third major objective of this study was to analyze the basis for the different cytoxic specificity of SLT-IIv as compared to Shiga toxin, SLT-I, and SLT-II. SLT-IIv did not bind to galactose-α1-4-galactose conjugated to bovine serum albumin, which is an analog of the eucaryotic cell receptor for Shiga toxin, SLT-I, and SLT-II. The results from the binding assays, taken together with the deduced amino acid comparisons between SLT-IIv and the other members of the Shiga toxin family, suggested that SLT-IIv binds to a different cellular receptor than do other members of the Shiga toxin family but has a similar mode of action. To test this

hypothesis, the A and B subunit genes of Shiga toxin and the SLTs were recombined by complementation and operon fusions so that hybrid toxins would be formed *in vivo*. In addition, specific amino acids in the B subunit of SLT-IIv were altered by site-directed mutagenesis to produce mutant cytotoxins. The activities of the hybrid and mutant cytotoxins were assessed in three ways: i) level of cytotoxicity, ii) ratio of HeLa to Vero cell cytotoxicity, and iii) ratio of extracellular to cell-associated cytotoxicity. The cytotoxic activities of the hybrid cytotoxins always corresponded to the activities of a native toxin possessing the same B subunit. These findings indicate that the B subunit of Shiga toxin and the SLTs dictates cell specificity and the ratio of extracellular to cell-associated cytotoxicity. While no single amino acid was identified as being essential for binding to a eucaryotic receptor, glutamine 64 in the B subunit of SLT-IIv was found to be critical for the extracellular localization of the toxin.

The fourth and final objective in this study was to create a non-toxinogenic strain which was isogenic to the parent strain *E. coli* S1191 so that the role of SLT-IIv in the pathogenesis of ED could be assessed. Although this strain was not constructed, several plasmids were derived that can be used to create such a strain in the future.

THE SHIGA AND SHIGA-LIKE CYTOTOXINS: GENETIC REGULATION AND FUNCTIONAL ANALYSIS OF THE BINDING SUBUNIT

by

Debra Lynne Weinstein

Dissertation submitted to
the Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1989

DEDICATION

I dedicate this dissertation, with love and respect, to the best husband in the world, Stafford Goldstein. I want this dissertation to be a testimony to our beautiful daughter, Rebecca, that you can achieve anything, if you work diligently and put forth your best effort. I hope that the example I am setting, like the examples set by my parents, instills in my children the understanding that the privilege of education is invaluable.

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INTRODUCTION

I. Preface

The introduction to this dissertation will be divided into three parts. Since extensive historical, structural and genetic details about Shiga toxin and Shiga like toxins type I (SLT-I) and II (SLT-II) have been the subjects of recent reviews by O'Brien and Holmes (1987) and Karmali (1989), the first part will provide only a general overview of these toxins. This first section will also include background information on one of the major topics of this dissertation, the regulation of Shiga and Shiga-like toxin production. Part two will provide a detailed historical perspective on the second major topic of this work, the Shiga-like toxin II variant (SLT-IIv) produced by edema-disease causing Escherichia coli. The third section will outline the specific aims of this dissertation.

II. Overview of Shiga toxin and Shiga-like toxins type I and II.

Historical perspective. Strains of Escherichia coli have been identified that cause intestinal disease in humans and animals. In a recent review, Levine (1987) defined four categories of diarrheagenic E. coli, which are summarized in Table 1. A fifth category, the enteroadherent E. coli (EAEC), has been described, but the role of EAEC in disease has not been proven. Entero-hemorraghic E. coli (EHEC), which is the category of E. coli studied in this project, cause hemorraghic colitis and/or the hemolytic uremic syndrome in humans. These E. coli are not invasive, do not produce the classical heat-stable or heat-labile

Table 1. The categories of diarrheagenic *Escherichia coli*[a].

Category	Acronym	Characteristic properties	Classical Disease association
Enterohemorrhagic	EHEC	non-invasive, produce Shiga-like toxins	hemorrhagic colitis hemolytic uremic syndrome
Enteropathogenic	EPEC	can be adherent, cause effacement of intestinal microvilli, harbor EPEC adherent factor (EAF)	infant diarrhea
Enterotoxigenic	ETEC	adherent, produce heat stable or heat labile toxins	traveler's diarrhea
Enteroinvasive	EIEC	invasive, resemble *Shigella*	dysentery

[a]taken from Levine, 1987.

enterotoxins, and most often belong to a restricted number of serotypes, including O157:H7, O26:H11, and O111:NM (reviewed in Levine 1987). EHEC produce elevated levels of cytotoxins that are identical or related to the Shiga toxin produced by Shigella dysenteriae type 1. Shiga toxin, which was first described in 1903 by Conradi, is considered to be one of the most potent of the bacterial toxins. These Shiga-like toxins (SLTs), which are sometimes referred to as Verotoxins (Konawalchuk et al., 1977), are functionally similar to Shiga toxin, i.e., they are paralytic and lethal for rabbits and mice, cause fluid accumulation in the ligated rabbit ileal loop model, and are cytotoxic for certain cell types in vitro (O'Brien et al., 1982; O'Brien et al., 1983; A.D. O'Brien, T.A. Lively, T.W. Chang, and S.L. Gorbach, Letter, Lancet ii:573 1983; A.D. O'Brien, T.A. Lively, M.E. Chen, S.W. Rothman, and S.B. Formal, Letter, Lancet i:702 1983). The SLTs have been categorized into two antigenically distinct types (Table 2): Shiga toxin and SLT-I are neutralized by polyclonal antisera to Shiga toxin and monoclonal antibody to the B subunit of SLT-I (Strockbine et al., 1985), whereas SLT-II is not neutralized by these antibodies.

The cytotoxins in the Shiga toxin family differ in both level (see Table 3 for the definition of low, moderate, and high levels of cytotoxin used by Marques et al., 1986, and throughout this dissertation) and distribution (cell-associated versus extracellular) of cytotoxic activity. Shiga toxin and SLT-I are associated with organisms that produce high levels of cytotoxin

Table 2. Summary of characteristics of Shiga toxin, SLT-I, and SLT-II[a].

Toxin	Level of cell-associated toxin (CD_{50}/ml)	Neutralized by			Location of structural genes	Disease association
		αST	MAbSLT-I	αSLT-II		
Shiga	~10^5 - 10^8	+	+	-	Chromosome	Bacillary dysentery
SLT-I	~10^5 - 10^8	+	+	-	Phage	Diarrhea, HC, HUS
SLT-II	~10^3 - 10^4	-	-	+	Phage	Diarrhea, HC, HUS

Abbreviations used: ST, Shiga toxin; HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome

[a]Taken from O'Brien and Holmes, 1987.

Table 3. Definition of cytotoxicity levels (CD_{50}/ml of sonic lysate)[a]

Level	Amount of cytotoxin produced (CD_{50}/ml of sonic lysate)
High	$1 \times 10^5 - 1 \times 10^8$
Moderate	$1 \times 10^3 - 1 \times 10^4$
Low	$2 \times 10^1 - 6 \times 10^2$

[a]Taken from Marques et al., 1986.

(Marques et al. 1986; Strockbine et al. 1986), while SLT-II is associated with organisms that produce moderate levels of cytotoxin (Strockbine et al., 1986). Shiga toxin is a periplasmic protein in S. dysenteriae type 1 (Donohue-Rolfe and Keusch, 1983; Griffin and Gemski, 1983) and is thought to be released into the media upon autolysis. Among EHEC that produce SLT-I or SLT-II, the levels of cell-associated SLT-I are about 1000-fold higher than for SLT-II (Strockbine et al., 1986), whereas the cytotoxic activity of SLT-II per ml of culture supernatant is greater than or equal to that of SLT-I. Therefore, SLT-I predominates in the cell-associated fraction (sonic lysates), and SLT-II predominates extracellularly (culture supernatants).

Role of toxins in disease. There is no direct proof that Shiga and Shiga-like toxins are virulence factors. However, circumstantial evidence implicates these toxins in disease. The possibility than these toxins play a role in disease is supported by the following findings. First, volunteers fed an invasive, chlorate-resistant, hypotoxinogenic mutant of S. dysenteriae type 1 developed a milder disease than volunteers fed the invasive, highly toxinogenic parent (Levine et al. 1973). This observation is consistent with the fact that S. dysenteriae type 1, which is the highest toxin producer of the shigellae, causes a more severe disease than other Shigella spp. (Prado et al., 1986). Second, monkeys fed wild type S. dysenteriae 1 had more severe colonic vascular lesions than monkeys fed an isogenic, toxin negative strain (Fontaine et al., 1988). Furthermore, blood was only seen

in the stools of monkeys fed the parental strain although all animals developed watery diarrhea.

Epidemiological data also supports a role for Shiga-like toxins in disease. Cleary et al. (1985) found that SLT-I was produced more commonly and in greater amounts by E. coli strains that were isolated from patients with diarrhea than from healthy individuals. Marques et al. (1986) found that 48 out of 49 strains producing moderate or high levels of SLT-I, SLT-II, or both toxins were associated with diarrhea, HC, or HUS. In contrast, only 1 out of 48 strains that produced elevated (i.e. moderate to high) levels of SLTs was isolated from healthy adults. The remaining 47 strains all produced low levels of a HeLa cell cytotoxin.

Structure. The structures of Shiga toxin, SLT-I, and SLT-II were described after the toxins were purified to homogeneity (Olsnes and Eiklid, 1980; Brown et al., 1982; O'Brien et al., 1980; O'Brien et al., 1982; O'Brien and LaVeck, 1983; Yutsudo et al., 1986; Pouche-Downes et al., 1987). The molecular weights of the holotoxins are approximately 70,000. Shiga toxin is known to be comprised of a single A subunit (32,000 MW) non-covalently linked to 5 copies of a B subunit (7,700 MW) (Donohue-Rolfe et al., 1984); the exact ratio of the A to B subunits in SLT-I or SLT-II has not been experimentally determined. The A subunit contains the enzymatic activity of the toxin (Reisbig et al., 1981; Donohue-Rolfe et al., 1984), and the B subunit is responsible for binding to a eucaryotic receptor (Olsnes et al., 1981; Donohue-Rolfe et al., 1984). Ito et al. (1988) recently separated the A and B

subunits of SLT-I and SLT-II and combined heterologous subunits to form cytotoxic hybrid molecules. These results demonstrate that the individual subunits of SLT-I and SLT-II can assemble to form fully cytotoxic molecules in vitro.

Receptor binding, internalization, and inhibition of protein synthesis. The eucaryotic receptor to which the B subunits of Shiga toxin, SLT-I, and SLT-II bind is a galactose-α1-4-galactose-containing glycolipid designated Gb_3 (Jacewicz et al., 1986; Lindberg et al., 1986; Lindberg et al., 1986; Lingwood et al., 1987; Waddell et al., 1988). The model developed by Keusch et al. (1982) to explain the path of Shiga toxin binding, entry, and intoxication is as follows. Shiga toxin binds to Gb_3 and then enters the cell by receptor-mediated endocytosis (Figure 1). A clathrin-coated pit is pinched off and a vesicle is formed. This acidic compartment promotes the cleavage of the A subunit, thereby creating an enzymatically active A_1 fragment. This A_1 fragment is delivered to the cytosol (by an unknown mechanism) where it binds the 60s ribosome.

Recent studies have revealed the exact mechanisms of action of the Shiga and Shiga-like toxins (Endo et al., 1987; Endo et al., 1988, Lamb et al., 1985; Figure 2). The A subunits of these toxins, like the plant toxin ricin, are N-glycosidases that cleave a specific adenine residue from the 28S subunit of eucaryotic ribosomal RNA. This cleavage results in the cessation of protein synthesis, and, ultimately, cell death. Montfert et al. (1987) determined the x-ray crystallographic structure of ricin and

Figure 1. Model for the receptor-mediated endocytic entry of Shiga toxin and processing of Shiga toxin in a mammalian cell (taken from O'Brien and Holmes, 1987, Figure 1, which was adapted from Keusch et al., 1982, Figure 6). The B subunit of the toxin binds to the mammalian cell receptor. Shiga toxin enters the cell by receptor-mediated endocytosis. The clathrin-coated pit is pinched off, and the coated vesicle is formed. The vesicle is acidified, and it may fuse with lysosomes. The mechanism by which the enzymatically active A_1 fragment of Shiga toxin is generated and reaches the cytosol is not known but is presumed to involve proteolytic nicking and reduction of disulfide bonds of the A subunit. The drawings above the dotted line represent the toxin molecule within the endocytic vesicle. The drawings below the dotted line represent the sequence of events once the A_1 fragment has been translocated into the cytosol (the mechanism of action of the A_1 fragment within the cytosol is depicted in Figure 2).

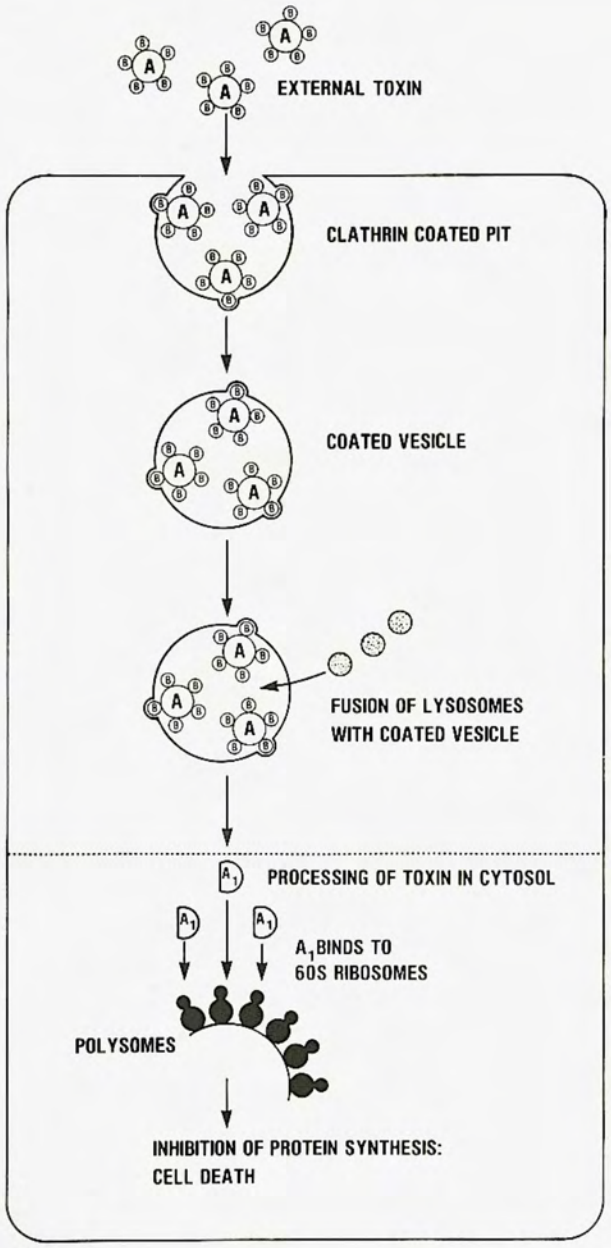
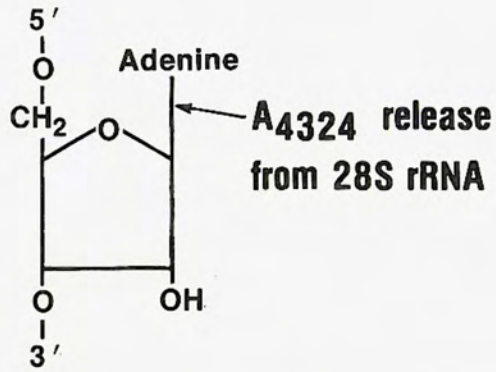


Figure 2. Mechanism of action of Shiga toxin, SLT-I, SLT-II, and ricin (taken from Endo et al., 1987). The enzymatically active portion of the toxin molecule (A_1) acts as an N-glycosidase to inactivate adenine residue 4324 in the 28S rRNA of rat liver ribosomes (or adenine residue 3732 in Xenopus oocyte ribosomes; Saxena et al., 1989). This N-glycosidase activity results in cessation of protein synthesis and cell death.

A SUBUNIT **MODE OF ACTION** *

Shiga toxin, SLT-I, SLT-II and ricin act as rRNA N-glycosidases:



Net result: Inhibition of EF-I dependent tRNAaa binding to 60S ribosomal subunit.

* Endo, et. al.

identified a prominent cleft in the A subunit which was assumed tocontain the active site. This information taken with a sequence comparison between the A subunits of SLT-I, SLT-II, and ricin was used by Hovde et al. (1988) to predict the active site residue of SLT-I. These investigators demonstrated that amino acid 167 (glutamic acid) in the A subunit of SLT-I is critical for the enzymatic activity of the toxin.

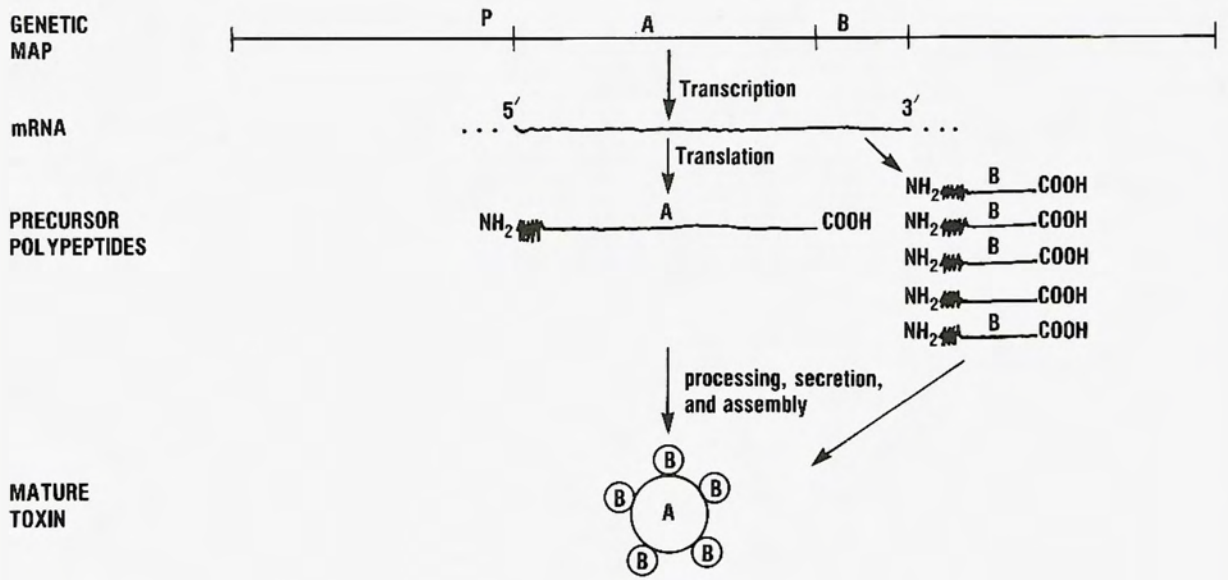
Genetics. The Shiga toxin operon, designated stx (Sekizaki et al., 1987), has been cloned from the chromosome of S. dysenteriae type 1 strain 3818T (Strockbine et al. 1988). The SLT-I and SLT-II operons, designated slt-I and slt-II respectively, have been cloned from toxin-converting coliphage (Newland et al. 1985; Willshaw et al. 1985; Huang et al. 1986; Newland et al. 1987; Kurazono et al., 1987). The nucleotide and deduced amino acid sequences of these three toxins have been determined and compared (Jackson et al. 1987; Jackson et al 1987a; Calderwood et al. 1987; DeGrandis et al. 1987; Strockbine et al. 1988). The nucleotide sequences of Shiga toxin and SLT-I are essentially identical; the toxins differ only by 3 nucleotides in the A subunit genes. These 3 nucleotide differences result in a single amino acid difference, a threonine at position 45 in the Shiga toxin A polypeptide, and a serine at the corresponding position in SLT-I (Strockbine et al. 1988). The nucleotide and deduced amino acid sequences of the B subunits of Shiga toxin and SLT-I are essentially identical (Strockbine et al. 1988.) These toxins and their structural genes are now referred to together as Shiga toxin/SLT-I and stx/slt-I,

respectively. By contrast, the structural genes for Shiga toxin/SLT-I share only 57-60% nucleotide sequence homology and 55-57% deduced amino acid sequence homology with SLT-II (Jackson et al. 1987a).

The stx/slt-I and slt-II operons are organized similarly. Each operon has a conserved promoter sequence 5' to the A subunit gene and conserved ribosome-binding sequences both 5' to the A subunit genes and in the untranslated space between the subunit genes (Figure 3). A possible mechanism for the translation of multiple copies of the B subunit for every A subunit is suggested by the organization of the operons; the independent ribosome-binding site for the B subunit may serve to enhance translation of the B subunit-coding sequence from the polycistronic mRNA. Newland et al. (1985) and Huang et al. (1986) demonstrated that the SLT-I B subunit is expressed in a clone lacking the A subunit gene which suggests the existence of a second promoter immediately 5' to the SLT-I B subunit gene.

Regulation of toxin production. In 1948, Dubos and Geiger studied the effects of different culture conditions on Shiga toxin production by S. dysenteriae type 1. These investigators were interested in the possible effects of iron on toxin production by S. dysenteriae type 1 because previous studies had demonstrated that the production of toxin by Corynebacterium diphtheriae and Clostridium tetani was inhibited by the presence of excess iron in the culture medium. Dubos and Geiger found that the optimum conditions for Shiga toxin production were aerobic growth in low

Figure 3. Working model of the organization and expression of the Shiga and Shiga-like toxin operons (taken from O'Brien and Holmes, 1987, Figure 2). The genetic map shows the location for the promoter (P) for the operons and the structural genes A and B. The mRNA is presumed to be polycistronic. The primary translation products correspond to the unprocessed A and B polypeptides of SLT-I which have amino-terminal signal sequences represented by the wavy lines. The oligomeric holotoxin is presumed to be assembled from the A and B polypeptides after they are secreted and processed to remove the signal sequences.

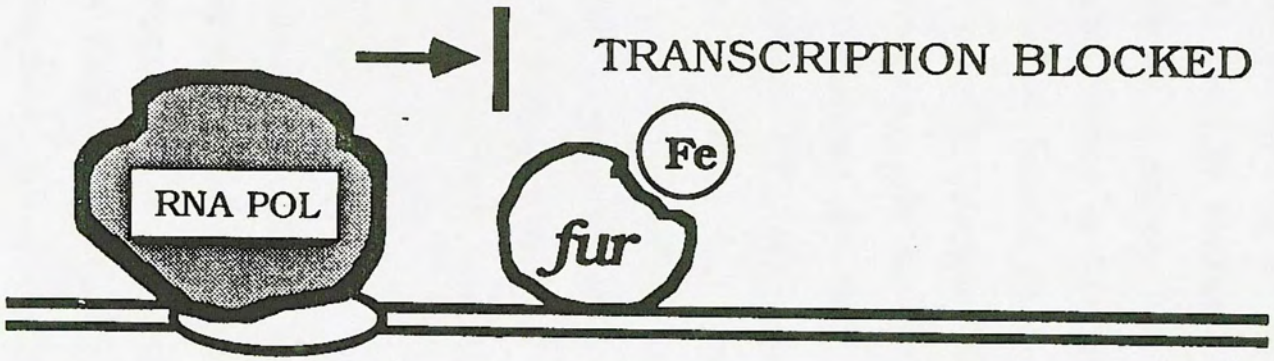


iron-containing media. Later studies confirmed that iron suppressed Shiga toxin synthesis (McIver et al., 1975, O'Brien and LaVeck 1982). O'Brien and LaVeck used iron-depleted media for production of SLT-I by E. coli but did not critically evaluate whether iron affected SLT-I synthesis. Therefore, one of the first goals of this project was to assess the effect of iron on SLT-I synthesis. Subsequently, when SLT-II and SLT-IIv were discovered (Strockbine et al. 1986; Marques et al. 1987), the effect of iron on production of these toxins was also evaluated.

Murphy et al. (1976) proposed a model for the iron regulation of diphtheria toxin at the genetic level. In this model, iron functions as a co-repressor with a C. diphtheriae cellular product to inhibit the transcription of the toxin genes. After the onset of this project, a similar model was proposed for the iron regulation of Shiga toxin/SLT-I synthesis (Figure 4) in S. dysenteriae 1 and E. coli. Betley et al. (1986) proposed that the E. coli fur gene product is involved in the iron regulation of slt-I genes. In this model, iron functions as a corepressor in conjunction with the fur gene product to bind specific operator sequences and inhibit the transcription of stx/slt-I.

Temperature plays an important role in regulating a set of genes required for virulence in Shigella spp. Maurelli et al. (1984) reported that several plasmid encoded genes which are critical for invasion are transcribed when the shigellae are grown at 37°C but not at 30°C. This observation led to the identification of a chromosomal locus, virR, which is proposed to encode a trans-

Figure 4. Model for the regulation of transcription of the stx/slt-I operon by the fur gene product (prepared by L. Sung, adapted from Betley et al., 1987). In this model, the fur gene product acts as a co-repressor with iron to bind a specific consensus sequence 5' to the coding region of the operon, thereby blocking the transcription of the operon by RNA polymerase (Calderwood et al., 1987a, 1988; DeGrandis et al., 1987).



acting repressor at the nonpermissive temperature. When this project was initiated, there had been no studies on regulation of Shiga or Shiga-like toxin production by temperature or virR.

II. The Shiga-like toxin type II variant.

History of the edema disease of swine. The edema disease of swine (ED) was first described by Shanks in 1938 as a disease that caused the sudden death of weanling (10-14 week old) pigs. The post-mortem characteristics of the disease included edema of the eyelids, wall of the stomach, and mesentery of the intestinal folds. Other characteristics of ED that have been noted since Shanks' original description include: neurological disturbances such as staggering gait, convulsions, limb paralysis, coma and death (Nielson 1981). In some cases, diarrhea is a prodrome of ED. Since Shanks' first report of this disease, ED has been recognized as a veterinary and agricultural problem; the morbidity in an infected herd is approximately 15% and the mortality of the affected pigs approaches 90% (Nielson, 1981). ED outbreaks have been reported in Europe, Asia, and North America, and the number of cases may be underestimated due to difficulty in establishing the diagnosis (Dr. Harley Moon, personal communication).

In 1950, Timoney reproduced the ED syndrome by inoculating weanling pigs with fluid from the intestinal contents of animals who had died from ED. In 1954, Schofield and Schroder ruled out the possibility that ED was caused by a preformed toxin in the feed. In 1957, Erskine et al. and Timoney, respectively, reproduced ED by intravenously inoculating swine with the cell-free

extracts of bacteria, and thus demonstrated that the etiology of ED was a bacterial product, not a viral agent. In 1960, Sojka et al. found that only a few serotypes of E. coli, primarily 0138, 0139, and 0141, were commonly associated with ED. In 1968, Smith and Halls reproduced ED by feeding E. coli that had been isolated from outbreaks of ED to susceptible pigs. This result proved that E. coli was the etiological agent of ED. Efforts to reproduce the clinical symptoms of ED with live E. coli or bacterial extracts have not been uniformly successful. One major problem has been the contamination of cell extracts with endotoxin; many of the symptoms seen in experimental ED can be explained by endotoxic shock (Clugston et al. 1974a). Another problem has been that only certain herds of swine appear susceptible to the effects caused by ED, which suggests that there may be some genetic predisposition of animals to ED. A recent study by Moon and his coworkers showed that while clinical symptoms of ED cannot always be experimentally reproduced, necrotic lesions in the vasculature of these animals are consistently found on autopsy (Dr. Harley Moon, personal communication).

The Edema disease principle/SLT-II variant. In 1969 Kurtz et al. described a biologically active substance produced by ED-causing E. coli which he called the edema disease principle (EDP). Subsequently, Clugston et al. (1974, 1974a) proposed that EDP might be responsible for the clinical manifestations of ED. Later, EDP was shown to share biological properties with Shiga toxin/SLT-I, i.e. it caused paralysis and death of pigs and mice (Gregory, 1960)

and killed Vero cells (Dobrescu, 1983). However, antibody to crude Shiga toxin/SLT-I did not neutralize the biological properties of EDP (Dobrescu, 1983, Marques, 1986). Furthermore, the neurological symptoms and histopathology seen in ED were characteristic of those caused by Shiga toxin; the time course was similar and the lesions seen in experimental animals were primarily in the vasculature of the spinal cord and brain (Bridgwater et al., 1955). A recent study by Francis et al. (1989) indicated that ED-like lesions could be induced in the brains of swine that were orally inoculated with an SLT-II-producing E. coli strain 0157:H7.

In 1983, Blanco et al. reported that EDP was more cytotoxic for Vero cells than for HeLa cells. It was not until the availability of immunological reagents for SLT-II that EDP was recognized to be an SLT and was renamed the SLT-II variant (SLT-IIv). SLT-IIv was classified by Marques et al. (1987) as a variant of SLT-II because it was neutralized by polyclonal antisera to SLT-II but, unlike other members of the Shiga toxin family, culture filtrates containing SLT-IIv killed Vero but not HeLa cells. The possibility that SLT-IIv may bind to a different receptor (or receptors) than Shiga toxin/SLT-I or SLT-II was first approached in the studies described herein.

Other differences between SLT-IIv and members of the Shiga toxin family are as follows. Monoclonal antibodies to the A subunit of SLT-II, which neutralize the cytotoxicity of SLT-II in vitro, show little or no neutralizing effects on SLT-IIv (Perera et al. 1988). This finding indicates that at least some of the

immunogenic epitopes of A subunits of SLT-II and SLT-IIv differ. Moreover, in contrast to SLT-II producing E. coli (O'Brien et al., 1984), no toxin-converting phage have been isolated from SLT-IIv producing ED strains (Marques et al., 1987). SLT-IIv, like SLT-II, is commonly associated with organisms producing moderate levels of cytotoxic activity.

The exact cause of the pathology observed in swine suffering from ED has not been determined. The endotoxin of E. coli is thought to contribute to some of the signs of ED. However, Clugston et al. (1974) separated endotoxin from crude EDP and determined that the edema and neurological signs seen in ED could not be reproduced by administering endotoxin alone. Some investigators believe that the symptoms of ED result from anaphylaxis caused by one or more unidentified E. coli proteins. However, anaphylactic shock is an immediate reaction and in experimental ED, the signs do not appear until 24-48 hours post-inoculation. That the cytotoxic effects of SLT-IIv (EDP) may contribute to the hypertension seen in ED was proposed by Clugston et al. (1974a). These investigators observed that approximately 40 hours following inoculation with EDP (endotoxin-free), the swine developed acute hypertension. Following the dramatic rise in blood pressure, the animals developed ataxia. Clugston et al. concluded that the hypertension caused by EDP (SLT-IIv) may lead to the neurological injury and death seen in ED.

III. Specific Aims.

The four major goals of this project were as follows. First,

mini-Mu lac operon fusions were constructed and used to confirm the organization of the slt-I operon and determine the effects of fusions within the promoter-proximal slt-I A gene on production of the slt-I B gene product. The effects of iron and temperature on the regulation of Shiga toxin and SLT-I production in S. dysenteriae type 1 strain 60R and strains of E. coli rendered toxinogenic either by lysogenization or transformation were studied. Also, the mini-Mu lac operon fusions were used to confirm the regulation by iron of the lac gene under control of the slt-I promoter. In addition, the effects of iron were assessed on the production of SLT-II and SLT-IIv in toxinogenic strains of E. coli. Second, the structural genes for SLT-IIv, slt-IIv A and slt-IIv B, were cloned from an edema-disease causing E. coli strain and the nucleotide sequences determined. The nucleotide sequences of the genes were compared with the nucleotide sequences of the genes encoding Shiga toxin/SLT-I and SLT-II. Third, the binding of SLT-IIv and SLT-II to a Shiga toxin/SLT-I eucaryotic cell receptor analog, galactose-α1-4-galactose conjugated to bovine serum albumin (Gal-Gal-BSA), was assessed. As an extension to the results from this study, hybrid cytotoxins were created which were comprised of the individual subunits of Shiga toxin/SLT-I, SLT-II, and SLT-IIv. The effects of a heterologous B subunit on cell specificity, levels of cytotoxicity, and the extracellular localization of the hybrid toxins were assessed. To determine residues in the SLT-IIv B subunit important for cell specificity or localization, oligonucleotide-directed site-specific mutagenesis was used to

change individual nucleotides in the B subunit gene. The levels of cytotoxic activity and extracellular localization of the mutant cytotoxins were assessed. Fourth, to determine whether SLT-IIv is responsible for or contributes to the signs of ED, attempts were made to create a nontoxinogenic strain which was isogenic for the parent edema-disease-causing strain, E. coli S1191. To create a non-toxinogenic mutant, a cloned SLT-IIv gene was inactivated by insertional mutagenesis and attempts were made to replace the wild type gene with the mutated gene.

METHODS AND MATERIALS

Bacterial strains and plasmids. The bacterial strains, plasmids, and bacteriophage used in this work are listed in Table 4.

Media, enzymes, biochemicals and radionuclides. Luria Broth (LB) or LB agar (Maniatis et al., 1982) was used for routine culturing of bacteria. For iron-regulation studies, Chelex (Bio-Rad Laboratories, Richmond, CA)-treated glucose syncase (O'Brien and LaVeck, 1983) was used with or without the addition of 10 μg/ml Fe^{3+} in the form of $FeCl_3$. Where indicated, media were supplemented with antibiotics (Sigma Chemical Co., St. Louis, Mo.) at the following concentrations: ampicillin (50 μg/ml), chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), tetracycline (12.5 μg/ml). Agarose for DNA electrophoresis was purchased from IBI (New Haven, Ct) or FMC Biochemicals (Rockland, Me.).

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL; Gaithersburg, Md.), Boehinger Mannheim Biochemicals (BM; Indianapolis, Ind.), or U.S. Biochemicals Corp. (Cleveland, Oh.). Nick translation and lambda packaging kits were purchased from BRL. DNA polymerase I (Klenow fragment), calf intestinal alkaline phosphatase (CIP), T4 DNA ligase and DNA kinase were purchased from Boehinger Mannheim. Sequenase DNA sequencing kit was purchased from U. S. Biochemicals Corp.

Radionuclides were purchased from either New England Nuclear Research Products (Boston, Ma.) or Amersham Corp.(Arlington Heights, Ill.).

Cytotoxicity and neutralization assays. Microcytoxicity assays

Table 4. Bacterial strains, plasmids, and bacteriophage used in this work.

Bacteriophage, strain, or plasmid	Description or genotype	Reference or source
Bacteriophage		
933J	SLT-I-converting	O'Brien *et al.*, 1984
933W	SLT-II-converting	Strockbine *et al.*, 1986
λvir	lytic phage λ	T. Maurelli
P1	lytic phage P1	T. Maurelli
Strains		
E. coli HB101	F⁻ *hsdS20*(r_B^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ⁻	Silhavy *et al.*, 1984
E. coli DH5α	F' *ϕ80dlacZ*, Δ(*lacZYA-argF*)U169, *recA1*, *endA1*, *hsdR17*(r_k^-, m_k^+), *supE44*, λ⁻, *thi-1*, *gyrA*, *relA1*	ibid.
E. coli JM109	*endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *traD36*, Δ (*lac proA,B*)/F', *proA,B*, $lacI^qZ$ M15	BRL
E. coli S1191	serotype 0139; produces SLT-IIv; Tc^r	Marques *et al.*, 1987
E. coli C600	⁻, *thi-1*, *thr-1*, *leuB6*, *lacY1*, *tonA21*, *supE44*, λ-	Silhavy *et al.*, 1984
E. coli C600(933J)	C600 with 933J; produces SLT-I	

Bacteriophage, strain, or plasmid	Description or genotype	Reference or source
Strains (continued)		
E. coli C600(933W)	C600 with 933W; produces SLT-II	
E. coli LE392	F'*supE*,*supF*,*hsdR*,*galK*,*trpR*,*metB*,*lacY*,*tonA*	Silhavy *et al.*, 1984
E. coli M8820TR(Mu*cts*)	F'*araD139*, Δ(*ara*-*leu*)7697,Δ(*proAB*-*argF*-*lacIPOZYA*)*XIII*,*rpsL*,*recA56*,*srl*::*Tn10*(Tc^r), Mu *cts*	Castilho *et al.*, 1984
E. coli POI1683	F'Mu dI1683 (Km^r),*ara*::Mu *cts3*,Δ(*proAB*-*argF*-*lacIPOZYA*)*XIII*,*rpsL*	ibid.
E. coli POI1681TR	POI1683 with Mu dI1681 (Km^r) in place of Mu dI1683 and *recA56*,*srl*::*Tn10*(Tc^r)	ibid.
E. coli MC4100	F'*araD139*,Δ(*argF*-*lac*),*U169*,*rpsL150*,*relA1*, *flbB5301*,*deoC1*,*ptsF25*,*rbsR*	Silhavy *et al.*, 1984
E. coli SY327 *pir*	Δ(*lac*-*pro*)*nalA*,*recA56*,*araD*,*argE*, RP4-2-tc::Muλ *pir*R6K	Miller *et al.*, 1988
E. coli CJ236	*dut*,*ung*,*thi*,*relA*	Bio-Rad

Bacteriophage

strain, or plasmid	Description or genotype	Reference or source
Strains (continued)		
S. dysenteriae 1 60R	produces Shiga toxin, non-invasive, rough	Dubos and Geiger, 1946
S. dysenteriae 1 BS242	strain 60R with virR::Tn10(Tc^r)	T. Maurelli
Plasmids		
pHC79	Tc^r,Ap^r cosmid vector	BM
pBR329	Tc^r,Ap^r,Cm^r; replicon ColE1	Covarrubias, 1981
M13 mp18 and mp19	sequencing vectors	BM
pACYC184	Cm^r,Tc^r, replicon P15A	Chang and Cohen, 1978
pBluescript SK	Ap^r, expression vector	Stratagene
pBluescribe	Ap^r, expression vector	Stratagene
pKSAC	kanamycin cassette	Pharmacia
pGP704	suicide vector; R6K replicon	J. Mekalanos
pRK2013	mobilizing vector; Km^r	Figurski and Helinki, 1979
pJN25	pBR328 with sltI; Ap^r	Newland et al., 1985
pNAS10	pBR329 with stx; Ap^r	Strockbine et al., 1988

Bacteriophage, strain, or plasmid	Description or genotype	Reference or source
Plasmids (continued)		
pNN103	pBR328 with slt-II; Ap^r	Newland et al.,1987
pMJ100	pBluescribe with slt-II; Ap^r	M. Jackson
pDLW5	pBR329 with slt-IIv; Ap^r	this study
pDLW5.3	pDLW5 with EcoR1 5'to slt-IIvA	this study
pDLW5.104	pDLW5 with EcoR1 in slt-IIv A	this study
pDLW5.1041	pDLW5.104 with Km^r cassette	this study
pDLW5.1042	insert from pDLW5.1041 in pACYC184;Cm^r,Km^r	this study
pDLW5.1043	insert from pDLW5.1041 in pGP704;Ap^r,Km^r	this study
pMJ153	pNAS10 with created EcoRV site;Ap^r	M. Jackson
pMJ330	pMJ100 with created Hpa1 site;Ap^r	M. Jackson
pDLW5.321	pDLW5.3 with created Hpa1 site;Ap^r	this study
pDW8	pBR328 with slt-I; mini-Mu in B subunit gene; Km^r,Ap^r	this study
pMJ331	pBluescribe with slt-II A from pMJ330;Ap^r	M. Jackson
pDLW101	pACYC184 with slt-I A; Cm^r	this study

Bacteriophage,

Strain, or plasmid	Description or genotype	Reference or source
Plasmids (continued)		
pJN26	pBR328 with slt-I B;Ap^r	Newland et al., 1985
pDLW102	pACYC184 with slt-II A;Cm^r	this study
pDLW103	pBluescript with slt-II B;Ap^r	this study
pDLW104	pACYC184 with sltII-v A;Tc^r	this study
pDLW105	pBluescript with sltII-v B;Ap^r	this study
pFUS1	ϕ(slt-IIv A-slt-II B) in pBR329;Ap^r	this study
pFUS2	ϕ(slt-II A-slt-IIv B) in pBR329;Ap^r	this study
pFUS3	ϕ(stx A-slt-II B) in pBR329;Ap^r	this study
pFUS4	ϕ(slt-II A-stx B) in pBR329;Ap^r	this study
pFUS5	ϕ(stx A-slt-IIv B) in pBR329;Ap^r	this study
pFUS6	ϕ(slt-IIv A-stx B) in pBR329;Ap^r	this study
pBMUTV1	B subunit mutation (Asn_{17} to Asp) in pBR329;Ap^r	this study
pBMUTV2	B subunit mutation (Gln_{64} to Glu) in pBR329; Ap^r	this study

Bacteriophage, Strain, or plasmid	Description or genotype	Reference or source
Plasmids (continued)		
pBMUTV3	B subunit mutation (Ile_{52} to Lys) in pBR329; Ap^r	this study
pBMUTV1.2	pBMUTV1 with Gln_{64} to Glu mutation	this study
pBMUTV2.3	pBMUTV2 with Ile_{52} to Lys mutation	this study

[a] Abbreviations used: Tc^r,Ap^r,Cm^r,Km^r, resistance to tetracycline, ampicillin, chloramphenicol, and kanamycin, respectively. stx, Shiga toxin operon; slt, Shiga-like toxin operon; A, A subunit gene; B, B subunit gene (of stx or slt); ϕ, operon fusion.

were done on Vero and HeLa cells according to modifications of the published methods of Gentry and Dalrymple (1980). Fifty ml overnight bacterial cultures were subjected to centrifugation for 5 min at 5000xg, and the supernatants were filter-sterilized and reserved. The pellets were washed twice with 0.85% NaCl (saline), then resuspended in 2.5 ml phosphate buffered saline (PBS; 0.15 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.5 mM KH_2PO_4; pH 7.6). The samples were subjected to sonication for 3 min with a pulse of 15 sec on, 10 sec off at 30 watts. The debris in the sonically disrupted bacteria (sonic lysates) was removed by centrifugation (9000xg for 20 min), and the supernatants were filter-sterilized. One hundred μl of ten-fold dilutions of the sonic lysates and the culture supernatants were incubated on HeLa or Vero cells for 48 hr at 37°C in a 5% CO_2 atmosphere. The surviving cells were fixed with 1.8% formalin and stained with 0.013% Coomassie Blue. The last dilution of the sample in which greater than or equal to 50% of the HeLa or Vero cells detached from the plastic as assessed by A_{620}, was considered the 50% cytotoxic dose (CD_{50}).

Toxin neutralization assays with polyclonal anti-SLT-II and anti-Shiga toxin or monoclonal anti-SLT-II A subunits (11E10 or 11F11; culture supernatants), anti-SLT-II B subunit (BC5; mouse ascites) and anti-SLT-I B subunit (13C4; culture supernatant) were done as described previously (O'Brien and LaVeck, 1983). Sonic lysates were prepared as above, and a series of six ten-fold dilutions were made in microtiter wells. One hundred μl of the appropriate antibody was added to 100 μl of each dilution and the

plates were incubated at 37°C for 1 hour, then at 4°C overnight. One hundred μl of each mixture was transferred to HeLa or Vero cells and the CD_{50} was determined as described above.

Preparation of plasmid and cellular DNA. Small and large scale preparations of plasmid DNA were obtained using methods outlined by Birnboim and Doly (1979) and Maniatis et al. (1982). For small scale plasmid preparations, the pellets from 1.5 ml overnight cultures were treated for 5 min with 2 μg/ml lysozyme in GTE (25 mM Tris, pH7.5, 10 mM EDTA, 50 mM glucose). The cultures were then treated for 5 minutes with 200 μl of 1% sodium dodecyl sulfate/0.2N NaOH (SDS/NaOH) on ice, followed by 150 μl of cold potassium acetate (3M potassium actetate, 2N acetic acid) for 5 minutes. The lysates were centrifuged, and the supernatants were extracted one time with phenol:chloroform:isoamyl alcohol (50:48:2), followed by ethanol precipitation (2 volumes cold ethanol were added followed by a 1 hour incubation at -20°C). The precipates were resuspended in 50 μl TE (50mM Tris pH 7.5, 10mM EDTA) containing 50 μg/ml DNAse-free RNAse. Ten μl of each preparation was digested with the appropriate restriction enzyme(s).

For large scale plasmid preparations, the pellets from 500 ml overnight cultures were treated for 5 minutes with 10 ml of lysozyme (5 mg/ml in GTE). The cells were then lysed with 20 ml SDS/NaOH for 10 min on ice, followed by 15 ml of ice cold 5M potassium acetate for 10 min. The lysates were centrifuged, and the supernatants were filtered through tissues. The filtrates were precipitated with isopropanol, centrifuged, and the pellets were

resuspended in 5 ml TE with 5.8 g cesium chloride and 0.6 ml ethidium bromide (EtBr; 10 mg/ml). The samples were calibrated to a refractive index of 1.392, then centrifuged in a Beckman VTi65.2 vertical rotor at 55,000 rpm for 20 hours. The plasmid bands were removed through the sides of the tubes with a 20-gauge needle, the EtBr was extracted with water-saturated butanol, and the samples were precipitated with ethanol twice. The DNA was finally resuspended in 500 μl of TE and stored at 4°C.

Total bacterial DNA was prepared according to modifications of methods described by Silhavy et al. (1984). Briefly, the pellets from 50 ml overnight cultures were treated for 30 min on ice with 1 ml lysozyme (1 mg/ml in GTE). The cells were then lysed with 0.5 ml 10% SDS, the lysates were treated with 100 μg proteinase K for 15 min at 56°C, and the samples were extracted one time with Tris-saturated phenol, two times with phenol:chloroform:isoamyl alcohol (50:48:2), then one time with chloroform:isoamyl alcohol (24:1). Following the extractions, two volumes of ice cold ethanol were added to each sample and the DNA was spooled out with a glass Pasteur pipette. The DNA was resuspended in 1 ml of TE and treated with 75 μg of DNAse-free RNAse for 30 min at 35°C. The samples were extracted once with phenol:chloroform:isoamyl alcohol (50:48:2), once with chloroform:isoamyl alcohol (24:1), then the DNA was precipitated with ethanol and spooled as before. The DNA was resuspended in 300 μl TE and stored at 4°C.

Transformation. Bacteria were made competent for transformation

with ligation mixtures or purified plasmid DNA using the $CaCl_2$ methods described by Hanahan (1983) or by electroporation methods described by the manufacturer (Bio-Rad). If the $CaCl_2$ method was used to render bacteria competent, overnight cultures of the bacteria to be transformed were diluted 1:100 in fresh LB and grown to mid-logarithmic phase. The cells were washed one time with 1/10 volume of 0.1M $MgCl_2$. The pellets were resuspended in 1/20 volume 0.1M $CaCl_2$ and incubated on ice for 15 min (for transformation of E. coli S1191, 15% dimethyl sulfoxide [DMSO] was added to the 0.1 M $CaCl_2$ solution). The cells were collected by centrifugation, resuspended in 1/50 volume cold 0.1M $CaCl_2$, and incubated for 12-24 hours on ice, at which time they were considered competent. Two hundred μl of competent cells were incubated for 2-5 hours on ice with DNA (ligation mixtures or purified plasmid DNA), heated for 2 min at 42°C (heat shock conditions), then diluted to 2 ml in LB. The transformants were then incubated at 37°C for 90 minutes and dilutions were spread on LB agar containing the appropriate antibiotic(s). To differentiate between lactose and non-lactose fermenting colonies, dilutions of bacteria transformed with either the pBluescribe or pBluescript vectors were spread on LB agar plates with 50 μl Bluo-gal (BRL; 2% in dimethyl formamide) and 25 μl IPTG (BRL; 100mM in water) on LB agar containing 50 μg/ml Ap.

When electroporation was used to render cells competent for transformation (Dower et al., 1988), overnight cultures of the cells were diluted 1:100 in fresh LB and grown to mid-logarithmic phase. The cells were washed one time with the electroporation

buffer (EP; 272 mM sucrose and 10% glycerol) and resuspended to 1/50 the original volume in EP. Four hundred μl of cells were mixed with 1-10 μg DNA on ice and immediately added to chilled electroporation cuvettes (Bio-Rad). Using the Bio-Rad gene pulser and pulse controller, the cells were administered 12.5 kv/msec. The cells were immediately diluted 10-fold with LB, incubated at 37°C for 90 minutes, and dilutions were plated on selective media.

Bacteriophage plaque assays. E. coli strain S1191 was tested for sensivity to infection by bacteriophage λ, P1, and M13 as a potential means of introducing DNA into the strain. E. coli HB101 served as a positive control. E. coli S1191 and HB101 were plated for plaque detection using 1% LB agar overlay containing approximately $2x10^8$ logarithmic phase E. coli. For experiments with λ, 1% maltose was added to the liquid growth media and 10mM $CaCl_2$ was added to liquid and solid media. For experiments with P1, 10mM $MgSO_4$ and 5 mM$CaCl_2$ were added to liquid and solid media. Serial 10-fold dilutions of the bacteriophage were made in LB with the appropriate additions. One μl of each dilution was spotted onto areas of the plate. The plates were incubated overnight at 37°C and scored for phage sensivity (appearance of plaques).

Conjugation. Conjugation was attempted as another potential means of introducing DNA into E. coli S1191 using a procedure described by Miller (1972). In this case, E. coli HB101(pDLW5) served as positive recipient control. Briefly, approximately 10^9 logarithmic recipient bacteria [strain S1191 or HB101(pDLW5)] and 10^9 donor bacteria [HB101(pRK2013)] were filtered onto a sterile 0.45 μm

filter (Millipore; Bedford, Mass). The filters were laid onto an L-agar plate and incubated at 37°C for 4-6 hours. The filters were washed with LB and dilutions were plated on selective medium (LB with Tc and Km for matings with S1191; LB with Ap and Km for matings with HB101[pDLW5]). The plates were incubated overnight at 37°C and scored for transconjugants.

Ampicillin/D-cycloserine cycling. One of the procedures used to create a nontoxinogenic strain of ED-causing strain E. coli S1191 in which the wild type slt-IIv gene had been replaced by a mutant slt-IIv gene was to select for the loss of the Cm^r plasmid vector and the maintenance of the antibiotic resistance which was unique to the cloned insert (Km^r). To select for transformants of E. coli S1191 which had lost their resistance to chloramphenicol, chloramphenicol-resistant cells were killed using a procedure described by Miller (1972). This method involves culturing bacteria in the presence of a bacteriostatic antibiotic (i.e. chloramphenicol) and then treating the cells with a bacteriocidal antibiotic that kills actively dividing cells (i.e. D-cycloserine or ampicillin). The bacteria that are killed are resistant to the bacteriostatic antibiotic (i.e. chloramphenicol-resistant) and the surviving organisms, which can be subcultured in non-selective media, are susceptible to the bacteriostatic antibiotic. Briefly, approximately 10^7 stationary phase cells were diluted in 5 mls of fresh LB with 250μg/ml of chloramphenicol and aerated at 37°C for 4-5 generations (approximately 3 hours). After the addition of 100 μg/ml of ampcillin and 2mM D-cycloserine, the aeration at 37°C was

continued until lysis was apparent (approximately 2 hours). The remaining cells were washed twice with fresh LB, then plated on LB agar.

Construction and characterization of mini-Mu transductants. Fusions derived by insertion of the transposable element mini-Mu lac into plasmid pJN25 were prepared by the methods described by Castilho et al. (1984). After confirming that strain POI1681TR(pJN25) and POI1683(pJN25) produced SLT-I, the strains were heat induced, and phage lysates were prepared. These lysates were used to infect the recipient strain M8820(Mu cts). Lactose-fermenting and lactose-nonfermenting transductants that contained fusion plasmids were identified as red and white colonies, respectively, on MacConkey lactose agar containing ampicillin and kanamycin.

To identify insertion mutants in which SLT-I synthesis was reduced or ablated, Km^r Ap^r mini-Mu lac transductants were grown in 1 ml of Luria broth overnight at 30°C, and filter-sterilized culture supernatants were tested for cytotoxicity. Cultures were grown at 30°C rather than 37°C to prevent thermal induction of Mu cts in the host strain E. coli M8820(Mu cts). Both undiluted culture supernatants and supernatants diluted 1:100 were tested in this screening assay to identify transductants that failed to release extracellular toxin or released less than did E. coli POI1681TR transformed with pJN25 and cultured under the same conditions.

Nontoxinogenic and hypotoxinogenic transductants were also

tested in the colony immunoblot assay to determine whether they produced the immunoreactive B subunit of SLT-I. For immunoblot tests, the transductants were grown at 30°C to prevent thermal induction of phage Mu functions. The immunoblot assay to detect production of the B subunit of SLT-I by individual bacterial colonies was performed as described by Strockbine et al. (1985), with minor modifications. Briefly, bacteria were grown on glucose-syncase agar for 48 h. The colonies were overlaid with nitrocellulose paper and then lysed with polymyxin B (Sigma Chemical Co., St. Louis, Mo.). After extensive washing of the paper with phosphate-buffered saline and blocking of the unreacted sites with gelatin, the blot was overlaid with monoclonal antibody specific for the SLT-I B subunit. The blot was again washed and then incubated with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories). After a final wash, the blots were developed with the substrate 0.05% (wt/vol) 4-chloro-1-naphthol and 0.015% (vol/vol) hydrogen peroxide for 30 min.

To test quantitatively for β-galactosidase activity, selected transductants were grown in glucose-syncase broth, and cells were lysed by chloroform-sodium dodecyl sulfate treatment. β-galactosidase assays were performed on the lysates as described by Miller (1972). The sites of insertion of mini-Mu into pJN25 in selected fusion plasmids were determined by restriction enzyme analysis of purified plasmid DNA.

Cloning and subcloning. Cosmid cloning and subsequent subcloning

were done as described by Maniatis et al. (1982). For cosmid cloning, total chromosomal DNA was partially digested with 0.1U of Sau3A1 per μg DNA for 7 minutes. The digested DNA was overlaid on a 5-20% continuous sucrose gradient and subjected to centrifugation at 40,000 rpm for 90 minutes. Fractions (400 μl) were collected and the size of the DNA in each fraction was determined by analyzing the electrophoretic mobility on a 0.7% agarose gel. The fractions containing predominantly 30-40 kb DNA fragments were pooled and ethanol precipitated. These fragments were ligated to the BamH1 digested cosmid vector, pHC79 by standard ligation conditions (20 mM Tris, pH7.6, 20 mM $MgCl_2$, 10 mM DTT, 0.6 mM ATP, 1U ligase/ug DNA at 15°C for 15-18 hr).

For routine cloning and subcloning, selected DNA samples and desired vectors were digested with the appropriate restriction enzymes for 2 to 24 hours under optimal conditions for the enzyme. The restriction enzyme(s) were inactivated either by heat treatment (65°C for 10 min) or phenol:chloroform:isoamyl alcohol (50:48:2) extraction, then the DNA was ethanol precipitated. The DNA sample was then used directly, treated with calf intestinal alkaline phosphatase (CIP; two treatments of 1U CIP/μg DNA in 50mM Tris, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine for 30 minutes each) or separated by electrophoresis on a 0.7% preparatory agarose gel. If the DNA was subjected to electrophoresis, the desired fragment was extracted by electroelution using the conditions described by the manufacturer of the electroelution apparatus (IBI). Fragments and vectors were ligated as described above.

Southern blot analysis. Southern blot hybridizations were done according to a modification of the method described by Southern et al. (1975). DNA samples were loaded into the wells of a 0.7% agarose gel and then separated by electrophoresis until the bromophenol blue dye marker migrated to the bottom of the gel. The gel was then treated for 10 min with 250 ml 0.25N HCl, followed by two 15 min treatments in 250 ml of 0.5N NaOH, 1.0 M NaCl. The gel was then treated twice for 30 minutes each time with a neutralizing solution (1M Tris, pH 8.0, 1.5 M NaCl). The gel was then placed on top of 12 sheets of Whatman 3 MM paper (Whatman Paper Ltd., England) cut to the same size as the gel and saturated with 20x SSPE (3M NaCl, 0.2M $NaH_2PO_4 \cdot H_2O$, 0.2M EDTA, pH7.4). A single piece of nitrocellulose paper (Shleicher and Schuell, Keene, New Hamp.) saturated with water was placed on top of the gel and overlaid with 7.5 cm of paper towels compressed with a 1 kg weight. The DNA was completely transferred to the nitrocellulose membrane after overnight incubation at room temperature. The nitrocellulose paper was then carefully removed, rinsed with 6x SSPE, and baked for 2 hr at 80°C under vacuum. The blot was soaked in prehybridization solution [6x SSPE, 0.5%SDS, 5x Denhardt's solution (Ficoll, 1% wt/vol; polyvinylpyrrolidone, 1% wt/vol; bovine serum albumin, 1% wt/vol; Sigma), 100 μg/ml sonicated salmon sperm DNA (Sigma)] for 2 hours at the hybridization temperature and then stored at 4°C.

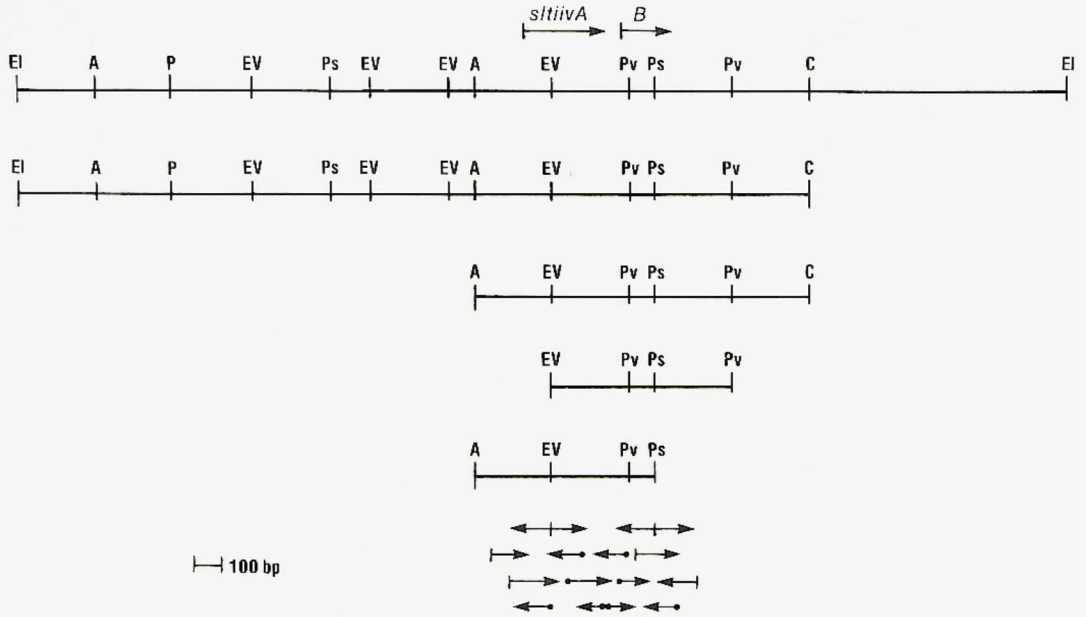
DNA was radiolabeled with ^{32}P-dCTP (NEN) by nick translation according to the supplier's instructions (BRL) and used to probe E. coli DNA. The probe was denatured by a 10 min treatment with

0.1N NaOH, neutralized with 0.18 M Tris-HCl/20mM Tris base, then added to the blot in hybrization solution (prehybridization solution with 0.01 M EDTA). After an overnight incubation at 65°C, the nitrocellulose membrane was washed at room temperature in 2xSSPE/0.2% SDS, followed by two washes in 1xSSPE/0.2% SDS at the desired temperature. The effect of temperature and sodium concentration on base pair mismatch was calculated by the following formula adapted from Maniatis et al. (1982):

% mismatch=(%G+C/2.44)+81.5-(600/length of probe in base pairs)-temperature+16.6xlog[Na^+]

Nucleotide sequence analysis. Fragments of the 4.2 kb AatII to ClaI insert in recombinant plasmid pDLW5 (Figure 5) were subcloned into the M13mp18 and mp19 replicative form vectors (Sanger et al., 1980). Either the M13 universal primer (New England Biolabs) or synthetic oligonucleotides prepared with a model 380A DNA synthesizer (Applied Biosystems Inc., Foster City, CA) were used as primers in the dideoxy chain terminator method (Biggin et al., 1983; Sanger et al., 1977) with a Sequenase kit (US Biochemicals Corp.) according to the methods provided by the supplier. The scheme used to determine the nucleotide sequence of the SLT-IIv structural genes is given in Figure 5. The MicroGenie computer program (Beckman Instruments, Inc., Palo Alto, CA) was used to analyze the slt-IIv sequence and compare it to the published sequences of stx/slt-I and slt-II. The IBI/Pustell DNA computer

Figure 5. Physical map of the slt-IIv operon. Restriction sites are indicated within the 11.6 kb EcoRI fragment isolated from the recombinant plasmid pDLW3 (cloned into plasmid vector pACYC184). Regions of the 11.6 kb EcoRI fragment subcloned into the vector pBR329 are shown, and their ability to produce biologically active cytotoxin on Vero cells is indicated on the right of the figure. The SLT-IIv structural genes, slt-IIv A and slt-IIv B, are oriented above the restriction map. The strategy used to sequence the coding and non-coding strands of slt-IIv A and slt-IIv B is depicted by the arrows below the map. Arrows preceded by a vertical line indicate subfragments sequenced using the M13 universal primer. Vertical lines that do not line up with demarcated restriction sites indicate RsaI sites (not labeled in this figure). Arrows preceded by a dot indicate where synthetic oligonucleotides specific to the slt-IIv sequence were used as primers. Restriction enzyme abbreviations are as follows: A, AatII; C, ClaI; EI, EcoRI; EV, EcoRV; Ps, PstI; Pv, PvuII.



Plasmid	Toxicity on Vero Cells
pDLW3	+
pDLW4	+
pDLW5	+
pDLW6	–
pDLW7	–

program (IBI, New Haven, CT) was used to estimate the isoelectric points and hydropathy plots of the deduced SLT-IIv A and B polypeptides.

Receptor binding assays. The procedure for the receptor-analogue binding ELISA was modified from the protocol of Lindberg et al. (1986). Two hundred μl of Galα1-4 Gal conjugated to bovine serum albumin (Gal-Gal-BSA; 2 μg/ml; Carbohydrates International, Chicago, IL) diluted in PBS (or PBS alone as a control) were used to coat a 96-well microtiter plate (Nunc; Kamstrup, Denmark) by overnight incubation at 4°C. Each well was then washed one time with PBS. One hundred μl of serial 5-fold dilutions of ammonium sulfate precipitated sonic lysates (O'Brien and LaVeck, 1982) from E. coli HB101(pDLW5) or E. coli C600(933W) were then added to the wells. The wells were washed four times with high salt PBS (0.5 M NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM NaN_3, 1% Tween-20; pH7.4), and 100 μl of a monoclonal antibody against the A subunit of SLT-II was then added to each well. This IgG_1 kappa monoclonal antibody, designated MAb11E10 (Perera et al., 1988), cross-reacts with SLT-IIv and also partially neutralizes its cytotoxicity. The plates were incubated at 37°C for 2 hours and then each well was washed 4 times with high salt PBS. One hundred μl of a 1 to 1500 dilution of horse-radish peroxidase conjugated to rabbit anti-mouse IgG and IgM (Boehringer Mannheim) was added to each well, and the plates were incubated at 37°C for 2 hours. Each well was then washed four times with high-salt PBS. One hundred μl of developing buffer (0.04 μg/ml of ortho-phenylenediamine and 0.45% hydrogen

peroxide in 33 mM citric acid, 67 mM Na_2HPO_4, pH5.0) was then added to each well, and the reaction was stopped after one hour by the addition of 15 μl of 8 N H_2SO_4. The absorbance of each well was then determined with a Titertek Multiskan MC reader (Flow Laboratories, Inc., McLean, VA) at 490nm.

Oligonucleotide-directed site-specific mutagenesis. The procedure for oligonucleotide-directed site-specific mutagenesis was modified from the protocol of Zoller and Smith (1984). Single-stranded M13 DNA containing the selected insert was purified from E. coli CJ236 using supplier's instructions (Bio-Rad). E. coli CJ236 carries mutations in the dut and ung genes, which result in the inactivation of dUTPase and uracil N-glycosylase, respectively. Consequently, the mutations result in high intracellular levels of dUTP, and DNA synthesized in E. coli CJ236 contains uracil in place of thymine. When single-stranded M13 DNA purified from E. coli CJ236 is transfected into a strain which does not carry the mutations in dut and ung (eg. JM109), the uracil-containing (wild type) strand is selectively degraded and the de novo strand containing the desired mutation is selected.

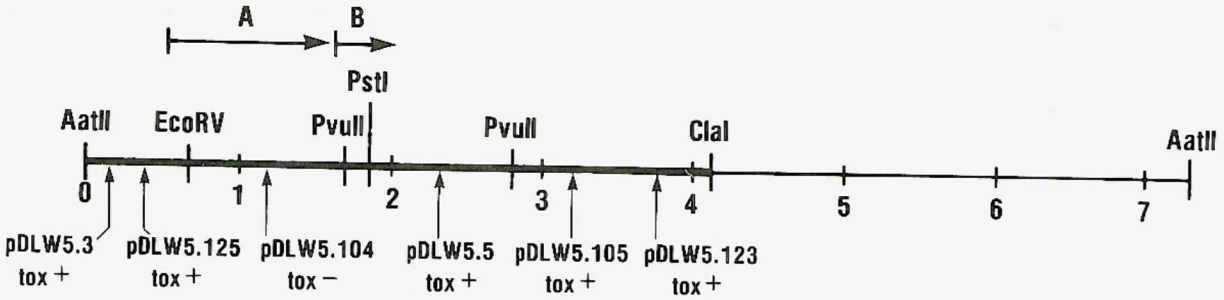
A synthetic oligonucleotide (25-30 mer) with only one nucleotide difference from the wild type toxin gene was annealed to the uracil-containing single-stranded DNA template. The second strand was synthesized from the oligonucleotide primer using DNA polymerase 1 (Klenow fragment) and deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP; Sigma). The 3' end of the de novo strand was ligated to the 5' end with DNA ligase. The ligation

mixtures were used to transfect E. coli JM109 and the transfectants were plated for plaque detection using 1% LB agar overlay containing approximately $2x10^8$ logarithmic phase E. coli JM109. The single-stranded DNA was prepared from transfectants and the nucleotide were sequences determined. Isolates containing the selected mutation were plaque purified and resequenced before being used for cloning.

Plasmid construction to create hybrid cytotoxins. Unique restriction sites that were useful for subcloning individual restriction fragments were created in plasmid pDLW5 by linker insertion and site-directed mutagenesis. Novel EcoR1 restriction sites were inserted within the RsaI sites of slt-IIv in pDLW5 (Figure 6) using 8-bp EcoRI linkers. These EcoR1 restriction sites were created within the toxin operon of the SLT-IIv-producing subclone pDLW5 using the following methods. Plasmid pDLW5 was partially digested with Rsa1 using the optimal conditions (0.1U enzyme/μg DNA at 37°C for 10 min) for obtaining single cut, linear fragments. The linearized DNA was then ligated to 5'-phosphorylated EcoR1 linkers (Pharmacia, Piscataway, New Jersey). The ligation mixture was digested to completion with EcoR1 and the mixture subjected to electrophoresis on a 0.7% preparative agarose gel. Linear sized fragments were excised from the gel, isolated by electroelution, precipitated with ethanol, and recircularized by ligation.

A single HpaI or EcoRV restriction site was created in the 12-15 nucleotide gaps between the A and B subunit genes of stx, slt-

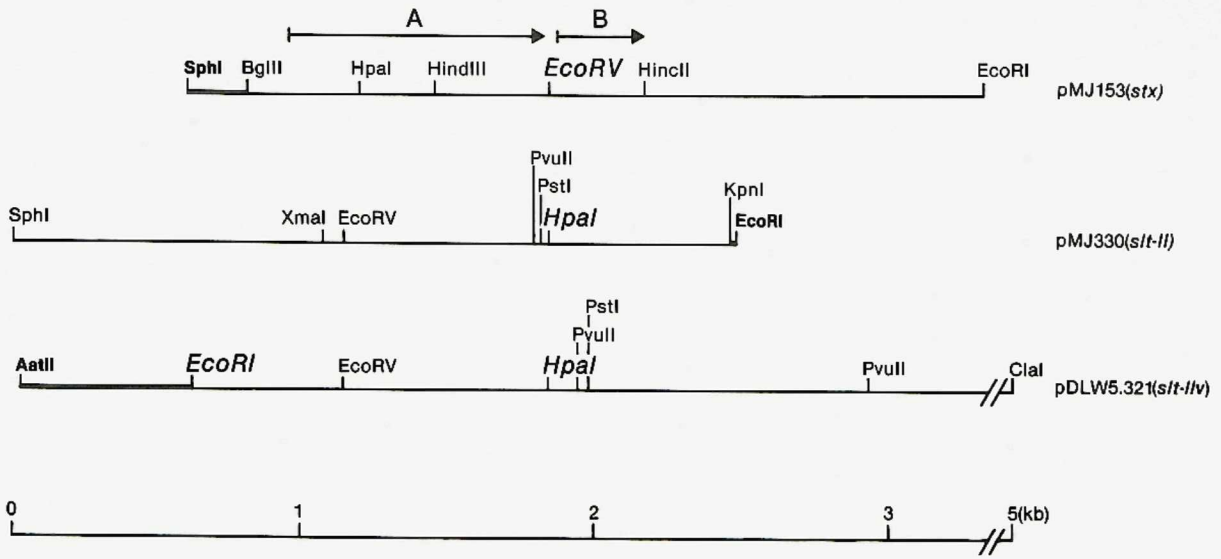
Figure 6. Physical map of the slt-IIv operon (pDLW5, which is cloned into plasmid vector pBR329) with EcoRI restriction sites created by linker insertion. Insert DNA is indicated in bold type. The A and B subunit structual genes are oriented above the restriction maps. The vertical arrows below the insert indicate the locations of the EcoRI restriction sites created by linker insertion. Map distances are given in kb. Abbreviations: tox^+ or tox^-, toxinogenicity or nontoxinogenicity or strains carrying the plasmid.



II, and slt-IIv using oligonucleotide-directed site-specific mutagenesis (Figure 7). The specific restriction site created ineach operon was selected for the following reasons: i) the restriction site did not naturally exist within the operon; ii) no more than three nucleotide changes were required for the creation of any site; iii) digestion at all of the created sites generated blunt ended DNA fragments which were compatible for the subsequent construction of operon fusions; and, iv) the changes did not alter the putative ribosome binding sequences nor any coding sequences for the B subunit genes. An EcoRV site was created in the 12 nucleotide gap between the A and B subunit genes of stx to create pMJ153 (Figure 7) by changing the following nucleotides: $guanine_{1113}$ to adenine, $adenine_{1116}$ to thymine, and $adenine_{1117}$ to cytosine (Strockbine et al., 1988). Plasmid pMJ100 was constructed by M. Jackson by cloning the 2.3-kb SphI-EcoRI fragment of slt-II from pNN103 (Newland et al., 1987) into the expression vector pBluescribe. A Hpa1 site was created in pMJ100 by changing a guanine to a cytosine in the gaps of slt-II at position 1204 (pMJ330) and slt-IIv at position 1212 (pDLW5.321; Figure 7 and Table 1; Jackson et al., 1987a; Weinstein et al., 1988). These sequence changes did not affect the expression of the toxin genes as determined by Vero cell cytotoxicity assays (data not shown).

Plasmids pDW8 and pMJ331 were used to construct A subunit subclones of SLT-I and SLT-II, respectively. Plasmid pDW8 (Weinstein et al., 1987; see Figure 9 in results section) carries an operon fusion which was derived by the insertion of a

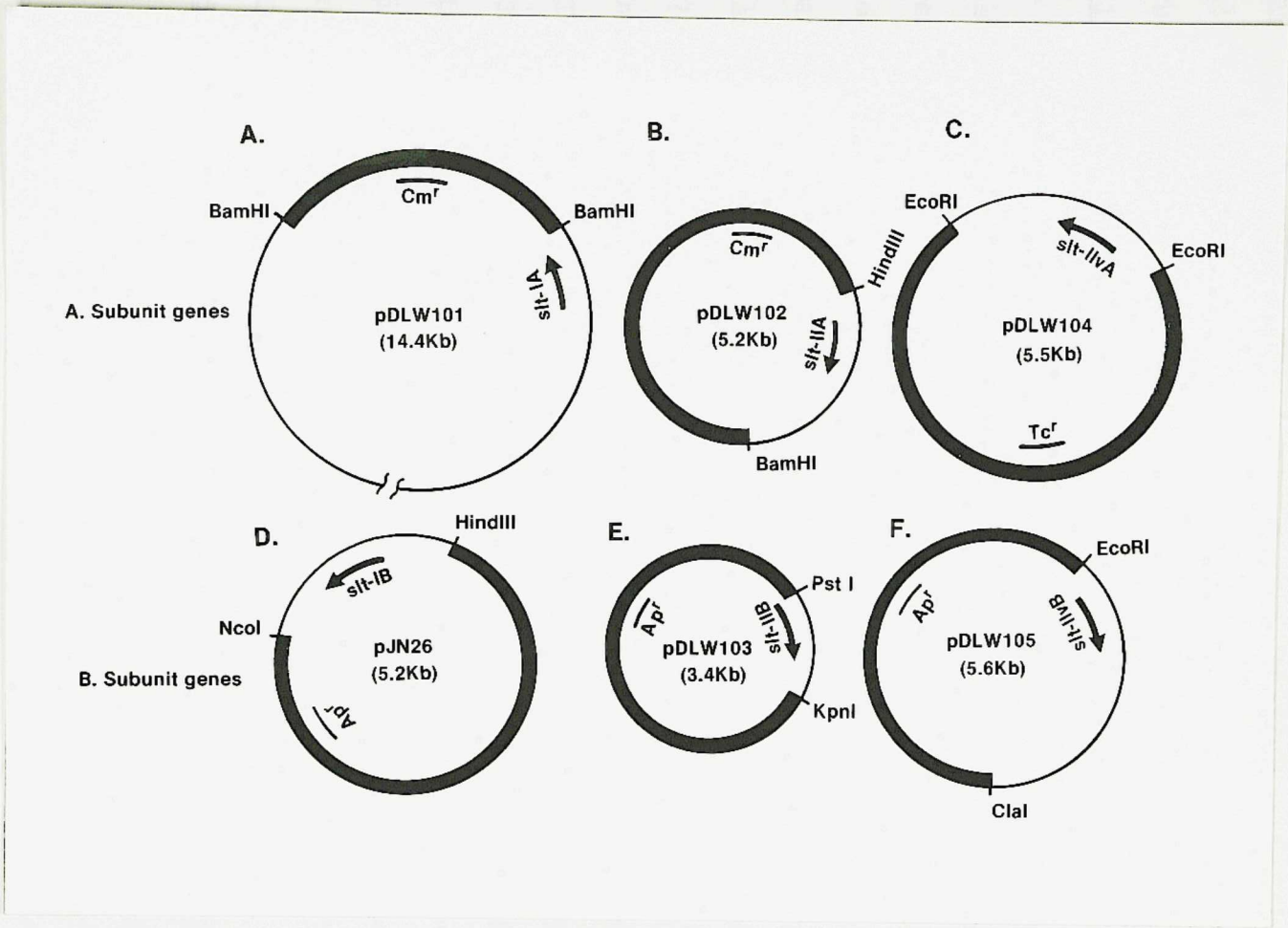
Figure 7. Physical map of the stx, slt-II and slt-IIv operons cloned into plasmids pMJ153, pMJ330, and pDLW5.321, respectively. The EcoRI restriction site created by linker insertion and the EcoRV and HpaI sites created by oligonucleotide-specific site-directed mutagenesis are indicated in italics. Vector DNA and restriction sites are indicated in bold type. The A and B subunit structural genes are oriented above the restriction maps. Map distances are given in kb.



transposable mini-Mu lac element into the slt-I B gene of plasmidpJN25 (Newland et al. 1985). Plasmid pMJ331 (created by M. Jackson), which carries the individual slt-II A subunit gene, was constructed by cloning the 1.8-kb SphI-HpaI restriction fragment from pMJ330 (Figure 7) into the SphI and HincII sites of the pBluescribe vector.

Complementation studies. For complementation studies, standard cloning and transformation procedures were performed as described above. To ensure the stable co-transformation of a single cell with two plasmids, one carrying an individual A and the other carrying an individual B subunit gene, the A and B subunit genes of each cytotoxin were cloned into plasmid vectors of different incompatibility groups (Figure 8). The A subunit genes of SLT-IIv, Shiga toxin/SLT-I, and SLT-II were cloned into pACYC184 which has a P15A replicon. The B subunit genes of the SLTs were cloned into either pBR328 or pBluescript SK, both of which have a ColE1 replicon. In previous studies, immunoprecipitation and colony blot assays have demonstrated that pJN26 (slt-I B subunit gene in pBR328) produces SLT-I B subunit (Newland et al., 1985; Weinstein et al., 1987; see Figure 10 in results section). The slt-IIv and slt-II B subunit genes were cloned into the expression vector pBluescript SK; hence, the genes are transcribed from a vector promoter. Individual A and B subunit encoding plasmids were co-transformed into E. coli HB101. Because the vectors used to clone the A and B subunit genes had different antibiotic resistance phenotypes, co-transformants were readily selected and maintained.

Figure 8. Physical maps of the plasmids carrying the individual A and B subunit genes used in complementation studies. To ensure the stable co-transformation of a single cell with two plasmids, one carrying an individual A and the other carrying an individual B subunit gene, the A and B subunit genes of each cytotoxin were cloned into plasmid vectors of different incompatibility groups. The restriction sites which flank the cloned inserts are indicated on each plasmid. Vector DNA is indicated by bold lines (vectors A-C, pACYC184; D, pBR328; E and F, pBluescript SK). The insert DNA was derived from the following plasmids: A, pDW8; B,pMJ331; C, pDLW5.3; D, pJN26 (Newland et al., 1985); E, pNN103 (Newland et al., 1987); F, pDLW5.104. The approximate locations of the subunit genes within the inserts are indicated by arrows. The abbreviations used are given in Table 4.



Sonic lysates and culture supernatants of co-transformants were tested for cytotoxicity on Vero and HeLa cells as described above.

Construction of operon fusions. Operon fusions containing the heterologous A and B subunit genes of stx, slt-II and slt-IIv were constructed. The A and B subunit genes from plasmids pMJ153 (stx/slt-I), pMJ330 (slt-II), and pDLW5.321 (slt-IIv) were isolated on the appropriate restriction fragments. Each fragment encoding an A subunit gene was then ligated to a fragment encoding a different B subunit gene and these six operon fusions cloned into plasmid vector pBR329. The individual restriction fragments used for creating the fusions were (Figure 7): i) the 1.3-kb SphI-EcoRV (stx/slt-I A) and 1.4-kb EcoRV-EcoRI (stx/slt-I B) fragments from pMJ153; ii) the 1.8-kb SphI-HpaI (slt-II A) and 0.5-kb HpaI-EcoRI fragment of pMJ330 (slt-II B); and, III) the 1.7-kb AatII-HpaI (slt-IIv A) and 2.3-kb HpaI-ClaI (slt-IIv B) fragments from pDLW5.321. The operon fusions, designated pFUS1-6, which are described in Table 1, were confirmed by nucleotide sequence analysis. The sonic lysates and culture supernatants of bacteria expressing each operon fusion were tested for cytotoxicity on Vero and HeLa cells as described above.

Plasmid construction for the creation of non-toxinogenic E. coli S1191. To create a nontoxinogenic variant of ED-causing E. coli S1191, a series of plasmids carrying the mutant, non-toxic slt-IIv gene were constructed. To construct a subclone which carried the mutant slt-IIv gene flanked by DNA which was homologous to the S1191 chromosomal DNA, the 4.1 kb AatII to ClaI fragment from

pDLW5.104 (Figure 6) was ligated to a 4.0 kb ClaI to EcoRI fragment from pDLW3 (Figure 5) into plasmid vector pBR329. To label this restriction fragment with a selectable marker, a 1.3 kb kanamycin resistance cassette was cloned into the EcoRI site which was internal to the SLT-IIv A subunit gene in pDLW5.104 to create pDLW5.1041 (Km^r, Ap^r). In order to create a Cm^r, Km^r, and Ap^s construct (pDLW5.1042) for use in D-cycloserine/ampicillin cycling experiments (using procedure described above), a 5.3 kb EcoRV fragment from pDLW5.1041 which carried the mutant toxin gene was cloned into pACYC184. This same fragment was also cloned into the suicide vector pGP704 to create pDLW5.1043.

Results

I. Regulation studies.

Organization and expression of the SLT-I operon. A set of mini-Mu lac operon fusions were constructed with plasmid pJN25 and used for studies on the organization and expression of the slt-I operon. A total of eight nontoxinogenic or hypotoxinogenic mini-Mu transductants were isolated, and the mini-Mu insertion sites were mapped (Figure 9). Seven of these transductants produced no detectable SLT-I, and one (pDW2) produced at least 100-fold less SLT-I than did the parent, both in supernatants and sonic lysates. Several additional toxinogenic mini-Mu transductants were mapped within the cloned insert, and the two located closest to the slt-I genes (pDW1 and pDW10) are shown in Figure 9.

To determine the direction of transcription of the slt-I genes, the lactose fermentation phenotypes of the eight nontoxinogenic or hypotoxinogenic mini-Mu insertions were noted on MacConkey lactose agar and compared with the orientation of the inserts (Figure 9). The lactose phenotypes were confirmed by performing β-galactosidase assays with cell extracts (Table 5). Five of the transductants were lactose fermenting, and the inserts in all five were oriented so that the lacZ gene was transcribed from left to right on the physical map shown in Figure 9. The other three transductants were lactose nonfermenting, and their inserts were oriented in the opposite direction.

Next, the transductants were tested in the immunoblot assay for immunoreactive B subunit determinants of SLT-I. The five

Figure 9. Locations of mini-Mu insertions into the EcoRV to NcoI insert of pJN25. The locations of the slt-I A and slt-I B genes and their transcriptional orientation as determined from nucleotide sequence analysis (Jackson et al., 1987) are shown above the map. Increments of 0.5 kilobases are indicated along the map. The vertical arrows below the insert indicate the insertion sites of mini-Mu. The horizontal arrows represent the orientation of the reading frame for the lacZ gene. Lac^+ or Lac^-, ability or inability of the strains carrying the plasmid to ferment lactose; tox^+,tox^-, or $tox^\pm$, toxinogenicity, nontoxinogenicity, or hypotoxinogenicity of strains carrying the plasmid; CB^+, CB^-, or $CB^\pm$, ability, inability, or partial ability of strains carrying the plasmid to produce immunoreactive SLT-I subunit B as assessed by colony blot.

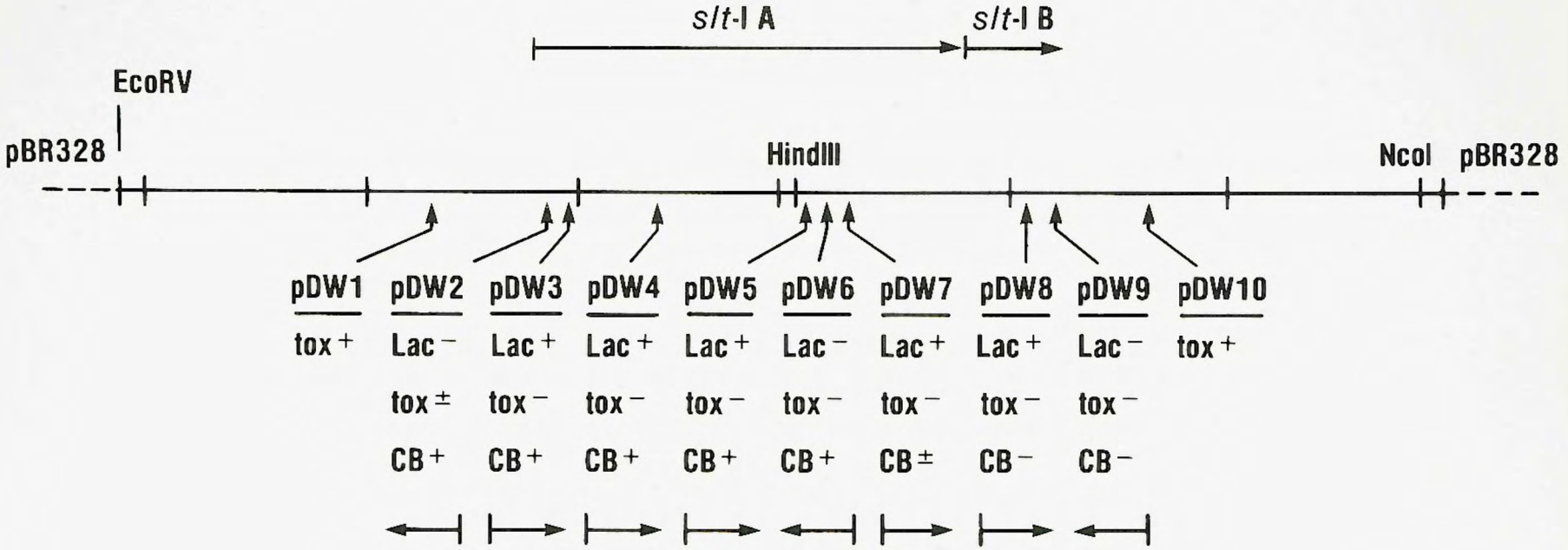


Table 5. Production of β-galactosidase by mini-Mu lac transductants.

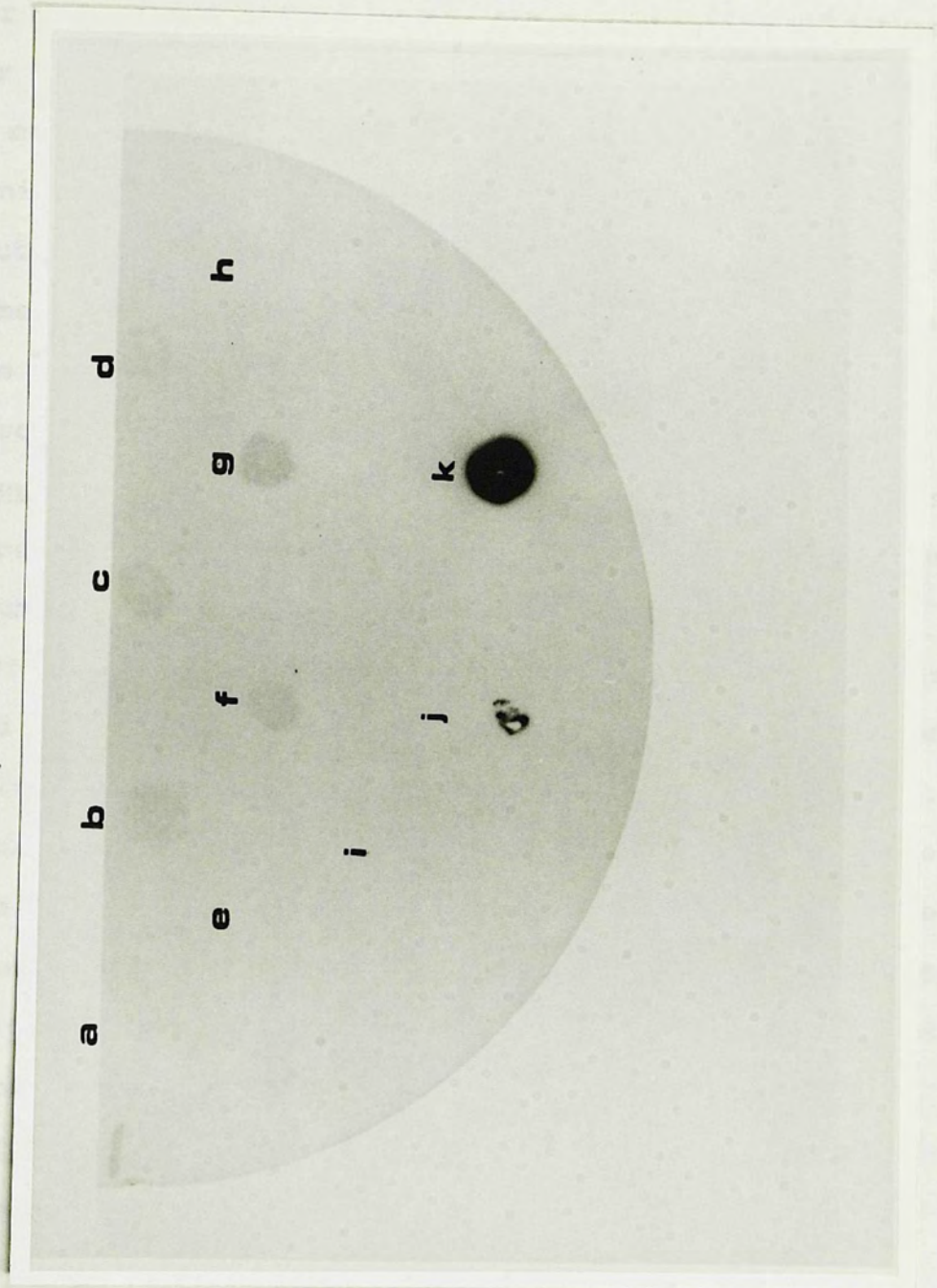
Fusion strain	Insertion location (subunit)	Lactose fermentation on MacConkey agar	Added iron	Amount of β-galactosidase (avg ± 2SEM)[a]
M8820(Mu cts)		-	-	20 ± 22
M8820(Mu cts) harboring plasmid:				
pJN25		-	-	13 ± 24
pDW2	A	-	-	24 ± 40
pDW3	A	+	-	3,697 ± 1,322[b]
pDW4	A	+	-	1,282 ± 485[b]
pDW5	A	+	-	3,036 ± 1,510[b]
pDW6	A	-	-	55 ± 53
pDW7	A	+	-	1,685 ± 704
pDW8	B	+	-	3,179 ± 1,628[b]
pDW9	B	-	-	120 ± 79
pDW3	A	+	-	4,570 ± 1,564[b]
pDW3	A	+	+	3,438 ± 1,280[b]

[a]One unit of enzyme produces 1 nmol of o-nitrophenol per min (Miller,1972). The results are based on data from five or six experiments.

[b]Statistically different from results of M88820(Mu cts) ($P<0.05$).

transductants which mapped to the left of pDW7 made easily detectable amounts of the SLT-I B subunit in this assay and were presumed to be located within the slt-I A gene (Figures 9 and 10). The two transductants which mapped to the right of pDW7 did not make detectable SLT-I B subunit and were therefore located within the slt-I B gene. The strain containing the pDW7 plasmid made very small amounts of immunoreactive B subunit. These findings located the slt-I A gene between the sites of the mini-Mu insertions in pDW1 and pDW8 and the slt-I B gene between the insertions in pDW7 and pDW10. These sites are consistent with the positions of the genes inferred from DNA sequence analysis (Jackson et al., 1987; Calderwood et al., 1987; DeGrandis et al., 1987). The observation that insertions of the mini-Mu lac element into the slt-I A gene eliminated toxicity but permitted expression of immunoreactive B subunit regardless of the orientation of the mini-Mu indicated that the SLT-I B subunit can be produced independently of the SLT-I A subunit. These results suggest that a promoter 5' to slt-I B is in the distal portion of slt-I A. The possibility that an independent promoter for slt-I B exists is consistent with the findings of Newland et al. (1985) and Huang et al. (1986), who showed by minicell analysis and in vitro transcription analysis, respectively, that clones lacking the slt-I A promoter could express the B subunit of SLT-I. However, these studies did not rule out the possiblity that a vector promoter is regulating the transcription of the individual slt-B gene.

Figure 10. Colony immunoblot assay (Strockbine et al., 1985) for detection of SLT-I B subunit in nontoxinogenic and hypotoxinogenic mini-Mu transductants. Blots for E. coli M8820(Mu cts) harboring the following plasmids; a, pDW7; b, pDW2; c, pDW4; d, pDW5; e, pDW9; f, pDW6; g, pDW3; h, pDW8; i, pJN28 (Newland et al., 1985), produces SLT-I A subunit only; j, culture supernatant from pJN25; k, pJN26 (Newland et al., 1985), produces SLT-I B subunit only. (The photographic reproduction does not show all of the positive colonies, so the colony blot results are summarized in Figure 9).



Iron regulation. The effects of iron on production of cytotoxin were determined for S. dysenteriae 1, E. coli that produce SLT-I (either by lysogenization with the SLT-I-converting wild-type phage 933J or by transformation with the multicopy hybrid plasmid pJN25, which carries the cloned slt-I structural genes), SLT-II (by lysogenization) or an SLT-IIv-producing wild type strain, S1191. The bacteria were grown in iron-depleted media with or without iron supplementation (200μM $FeCl_3$), and cytotoxin levels were determined (Tables 6 and 7).

Iron significantly suppressed total toxin synthesis by S. dysenteriae 1 strain 60R grown at 37°C, as expected based on previous studies (Dubos and Geiger, 1946; van Heyningen and Gladstone, 1953; McIver et al., 1975; O'Brien and LaVeck, 1982). The effect was more dramatic when the total toxin units were divided by bacterial growth (A_{600}) because the 60R cultures without added iron grew less well than the cultures with added iron. Iron also suppressed total toxin production by the SLT-I producing lysogen E. coli C600(933J) grown at 37°C but had less influence on the growth of strain C600(933J) than on that of S. dysenteriae 1 strain 60R. No statistically significant difference in total toxin was observed when iron was added to cultures of E. coli MC4100(pJN25) grown at 37°C nor did the A_{600} differ in the presence or absence of added iron. However, as shown in Table 6, significantly more toxin was produced at 37°C by E. coli MC4100(pJN25) than by either S. dysenteriae 1 strain 60R or E. coli C600(933J).

Table 6. Effects of iron and temperature on Shiga toxin and SLT-I production.

Organism (n^b) (type of toxin produced)	Added iron	Temp (°C)	A_{600} (mean ±2 SEM)	Amount of cytotoxin (mean $\log_{10}$ ±2 SEM)[a]		
				Cell associated	Extracellular	Total/A_{600}
S. dysenteriae 1 60R (3)	-	37	3.3 ± 0.4	7.8 ± 0.5	8.1 ± 0.8	8.1 ± 0.1
(Shiga toxin)	+	37	6.7 ± 1.6[c]	7.1 ± 0.3	6.5 ± 0.4[c]	6.5 ± 0.2[c]
	-	30	3.2 ± 0.3	7.6 ± 0.1	6.7 ± 0.0[d]	7.1 ± 0.1[d]
	+	30	5.9 ± 0.2[c]	6.9 ± 0.4	6.1 ± 0.0[c]	6.5 ± 0.1[c]
E. coli C600(933J) (8)	-	37	3.4 ± 0.7	8.2 ± 0.3	6.7 ± 0.3	7.7 ± 0.4
(SLT-I)	+	37	4.3 ± 0.8	7.2 ± 0.3[c]	6.6 ± 0.2	6.7 ± 0.3[c]
(7)	-	30	1.9 ± 0.3[d]	7.6 ± 0.1[d]	5.5 ± 0.2[d]	7.3 ± 0.2
	+	30	4.0 ± 0.9[c]	7.4 ± 0.3	5.1 ± 0.2[c,d]	6.8 ± 0.2[c]
E. coli MC4100(pJN25) (7)	-	37	4.3 ± 0.3	9.7 ± 0.4	8.5 ± 0.7	9.2 ± 0.4
(SLT-I)	+	37	4.3 ± 0.3	9.5 ± 0.2	7.7 ± 0.6	8.9 ± 0.2
(4)	-	30	3.4 ± 0.3[d]	9.3 ± 0.2	8.7 ± 0.0	8.9 ± 0.2
	+	30	5.8 ± 0.9[c,d]	9.4 ± 0.1	6.5 ± 0.2[c,d]	8.6 ± 0.0[c]
E. coli POI1681TR(pJN25) (5)	-	30	2.3 ± 0.0	8.9 ± 0.2	7.6 ± 0.4	8.5 ± 0.2
(SLT-I)	+	30	2.7 ± 0.4	8.7 ± 0.7	7.4 ± 0.5	8.4 ± 0.7

[a]Cell-associated, $\log_{10}CD_{50}$ per pellet; extracellular, $\log_{10}CD_{50}$/50 ml of supernatant; total, cell-associated CD_{50} plus extracellular CD_{50}/A_{600}.

[b]Number of samples.

[c]Significantly different ($P<0.05$) by Student's unpaired t test from value for the same strain grown at the same temperature without added iron.

[d]Significantly different ($P<0.05$) by Student's unpaired t test from value for the same strain grown at 37°C under the same conditions of added iron.

Table 7. Effects of iron on SLT-IIv, SLT-II, and SLT-I production.

Organism[b] (type of toxin produced)	Added iron	A600 (mean ±2 SEM)	Amount of cytotoxin (mean $\log_{10}$±2 SEM)[a]		
			Cell associated	Extracellular	Total/A_{600}
E. coli S1191	-	3.9±0.6	4.7±0.2	5.2±0.4	4.6±0.3
(SLT-IIv)	+	4.9±0.3[c]	5.7±0.9	5.1±0.7	5.1±0.7
E. coli C600(933W)	-	2.7±0.9	5.2±0.1	6.5±0.6	5.9±0.4
(SLT-II)	+	2.7±0.6	5.4±0.2	6.5±0.7	5.9±0.5
E. coli C600(933J)	-	3.0±0.5	8.0±0.3	6.6±0.5	7.5±0.3
(SLT-I)	+	4.5±0.7[c]	6.7±0.7[c]	6.3±0.2	6.3±0.4[c]

[a]Cell associated, CD_{50} per pellet; extracellular, CD_{50}/40 ml supernatant; total, cell-associated CD_{50} plus extracellular CD_{50}.

[b]Each group consisted of 4 samples. E. coli S1191 was tested for cytotoxicity on Vero cells, while E. coli strains C600(933J) and C600(933W) were for cytotoxicity on HeLa cells. In experiments using E. coli S1191, E. coli C600(933J) was tested on Vero cells as a positive control for iron regulation. The CD_{50}'s of E. coli C600(933J) on Vero cells were not significantly different from the CD_{50}'s of E. coli C600(933J)on HeLa cells by Student's unpaired t test.

[c]Significantly different ($P<0.05$) by Student's unpaired t test from value for the same strain grown without added iron.

At 30°C the addition of iron to cultures of S. dysenteriae 1 strain 60R and E. coli C600(933J) suppressed total toxin synthesis per A_{600} unit (Table 6). For S. dysenteriae 1, iron also significantly suppressed total toxin production. In contrast, for E. coli C600(933J), MC4100(pJN25), and POI1681TR(pJN25) at 30°C, iron did not affect total toxin synthesis (Table 6). E. coli C600(933J) and MC4100(pJN25) grew to significantly higher A_{600} values in cultures with added iron, and for these two strains the total toxin per A_{600} was significantly less with added iron. For strain POI1681TR(pJN25), the effect of iron on toxin production at 37°C could not be determined because it is lysogenized with a thermally inducible Mu phage (Castilho et al., 1984).

The effects of high and low iron concentrations on the β-galactosidase activity in the lactose-fermenting mini-Mu operon fusions were also examined. The results of several experiments with pDW3, the lactose-fermenting insert closest to the promoter 5' of slt-I A (Figure 9), are given in Table 5. No significant differences were noted in the levels of β-galactosidase produced by pDW3 under high- or low-iron conditions. Similar results were found with the other lactose-fermenting insertion mutants (data not shown). Therefore, the effect of iron on SLT-I production can not be distinguished in the mini-Mu transductants.

Iron had no significant effect on the production of SLT-IIv or SLT-II (Table 7). Therefore, members of the Shiga toxin family differ in the mechanism(s) of toxin regulation. A recognition site for the E. coli fur gene product (Fur) has been located in the

promoter regions for the stx operon (Strockbine et al., 1988) and the slt-I operon (Calderwood et al., 1987; Calderwood and Mekalanos, 1987a, 1988; DeGrandis et al., 1987). Fur protein may function with iron as a co-repressor to negatively regulate toxin production (Figure 4; Betley et al., 1986). Neither the putative promoter sequences of slt-IIv (from sequence data, see Weinstein et al., 1988; Gyles et al., 1988) nor slt-II showed any significant homology to a binding site for the fur gene product (Sung, L., D. Weinstein, M. Jackson, A. D. O'Brien, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1988. D77, p.84).

Temperature regulation. The effects of temperature on Shiga toxin and SLT-I production were evaluated by comparing cytotoxin production of S. dysenteriae 1 and strains of E. coli that had been rendered toxinogenic either by lysogenization with phage 933J or by transformation with pJN25, grown in identical media at different temperatures. At 37°C without added iron, S. dysenteriae 1 strain 60R produced significantly larger amounts of total toxin and toxin per A_{600} unit than at 30°C (Table 6). In contrast, the effect of temperature on cultures of S. dysenteriae 1 with added iron was not statistically significant. Therefore, the effects of higher temperature and iron deprivation on toxin production by S. dysenteriae 1 are additive. In other words, the optimal conditions for Shiga toxin production by S. dysenteriae 1 are at 37°C in iron-depleted media. With E. coli, temperature did not significantly affect the total amount of toxin produced per A_{600} unit for any of the strains tested (data not shown). For E. coli C600(933J) grown

in medium without added iron, the total amount of toxin produced at 37°C was significantly greater than at 30°C, but this difference was entirely accounted for by the greater growth at 37°C.

The virR gene in Shigella spp. is known to affect the temperature regulation of several virulence genes; the virulence genes are expressed at 37°C and not at 30°C (Maurelli et al., 1984). To analyze the effects of virR on the temperature regulation of Shiga toxin in S. dysenteriae type 1, cytotoxin production was compared in isogenic strains which differed only in the virR gene (Table 8). S. dysenteriae type 1 strain 60R has a wild type virR gene, whereas the virR gene in strain BS242 has been inactivated by transposon mutagenesis. At 37°C, both the wild type and virR- mutant strains of S. dysenteriae 1 produced significantly larger amounts of total toxin and toxin per A_{600} unit than at 30°C (Table 8). Therefore, virR does not influence the temperature regulation of Shiga toxin production in S. dysenteriae 1 strain 60R.

II. Cloning and sequencing of the SLT-II variant genes.

Cloning of toxin genes. The operon that encodes SLT-IIv was cloned from a partial Sau3A digest of the total cellular DNA of E. coli S1191 into the BamHI site of cosmid vector pHC79. Transductants were screened for biological activity as assayed in the Vero cell cytotoxicity assay. Two of 800 transductants screened produced Vero cell cytotoxins that were not active on HeLa cells. The Vero cell cytotoxins produced from these two transductants were neutralized by polyclonal antisera to SLT-II but not by rabbit anti-Shiga toxin. These two transductants carried recombinant

Table 8. Effects of the virR gene on the temperature regulation of Shiga toxin production in S. dysenteriae type 1.

Strain of[b] S. dysenteriae 1 (genotype)	Temp. °C	A600 (mean)	Amount of cytotoxin (mean $\log_{10}$)[a] Cell associated	Extracellular	Total/ A_{600}
BS242	37	1.5	8.2	7.9	8.2
(virR$^-$)	30	1.6	7.4	7.2	7.5
60R	37	2.9	7.7	7.5	7.6
(virR$^+$)	30	2.1	7.2	6.9	7.1

[a]Cell associated, CD_{50} per pellet; extracellular, CD_{50}/40 ml supernatant; total, cell-associated CD_{50} plus extracellular CD_{50}/A_{600}.

[b]Each value represents the mean of 2 samples.

plasmids designated pDLW1 and pDLW2 that were presumed to carry the intact slt-IIv operon.

Digestion of plasmid DNAs from pDLW1 and pDLW2 with EcoRI, BamHI, and HindIII revealed common restriction fragments. Subsequent subculturing of E. coli harboring pDLW1 or pDLW2 revealed that pDLW1 was not maintained stably within the cells. Therefore, pDLW2 was selected for further subcloning. Purified pDLW2 was digested to completion with EcoRI and the fragments randomly subcloned into the vector pACYC184. A recombinant plasmid isolated from an SLT-IIv producing subclone (pDLW3) carried an 11.6 kb EcoRI insert (Figure 5). To identify more precisely the physical location of the slt-IIv genes, I constructed a series of subclones in plasmid vector pBR329 and examined their toxin phenotypes on Vero cells (Figure 5). Only subclones that contained the 4.1 kb AatII to ClaI restriction fragment gave positive toxin results. Based on these results, it was concluded that the SLT-IIv genes were cloned from ED-causing E. coli S1191.

Determination of the location of toxin genes in E. coli S1191. Southern hybridization analyses were used to study the location of the SLT-IIv genes within the genome of E. coli S1191. Although no SLT-IIv-converting coliphage were isolated from E. coli S1191 in a previous study (Marques et al., 1987), whole cell DNA was examined for the presence of phage sequences related to the SLT-I-converting phage 933J and the SLT-II-converting phage 933W (O'Brien et al., 1984). Whole cell DNA from E. coli strains S1191, C600, as well as the lysogens C600(933W) and C600(933J), were probed with

radiolabeled 933W or 933J phage DNA. Under hybridization conditions that allowed up to 40% base pair mismatch, no distinct bands of E. coli S1191 DNA hybridized to either the 933W or 933J phage probes (Figure 11). The data from these blots confirm that the 933J and 933W phage share some homology (Figure 11, lanes 4 and 5; Strockbine et al., 1986).

The presence of plasmids in E. coli S1191 was demonstrated by cesium chloride purification of plasmid DNA and 0.7% agarose gel electrophoresis followed by ethidium bromide staining (Figure 12, panel A, lane 1). To assess whether the SLT-IIv genes were located on any of these plasmids or on the chromosome, purified plasmid DNA from E. coli S1191 as well as EcoRI-digested whole cell DNAs from E. coli S1191, E. coli C600(933W), and E. coli C600(933J) were probed with the radiolabeled 1.06 kb EcoRV to PstI fragment from pDLW5 (Figure 12, panel B). This probe, which is internal to the slt-IIv operon (Figure 5), hybridized to a single 11.6 kb EcoRI band in the whole cell DNA of E. coli S1191 and a 4.9 kb EcoRI fragment from the whole cell DNA of E. coli C600(933W) (Figure 12 lanes 3 and 4). The slt-II genes of phage 933W are known to be located on a 4.9 kb EcoRI fragment (Newland et al., 1987). Under these hybridization conditions, the probe did not hybridize to any of the plasmids isolated from strain S1191 (Figure 12 lanes 1 and 2) nor to the whole cell DNA of E. coli strains C600(933J) and C600 (Figure 12 lanes 5 and 6). These findings indicate that the slt-IIv genes are on the chromosome of E. coli S1191 and are homologous to the SLT-II genes.

Figure 11. Southern blot analysis of cesium chloride purified plasmid DNA and EcoRI-digested plasmid and whole cell DNA samples. Panel A shows the autoradiograph of a Southern blot after hybridization with ^{32}P-labeled phage 933J DNA (O'Brien et al., 1984). Panel B shows the autoradiograph of a Southern blot after hybridization with ^{32}P-labeled phage 933W DNA (O'Brien et al., 1984). Hybridization conditions allowed for 40% base pair mismatch. The autoradiograph represents a 24 hour exposure in the presence of an intensifying screen (Eastman Kodak, Co., Rochester, N.Y.). Lanes 1, cesium chloride purified plasmid preparation from E. coli S1191; lanes 2, cesium chloride purified plasmid preparation from E. coli S1191 digested to completion with EcoR1; lanes 3-6, whole cell DNA isolated from E. coli S1191 (lanes 3), E. coli C600(933W) (lanes 4), E. coli C600(933J) (lanes 5) and E. coli C600 (lanes 6). Numbers on the left indicate sizes in kilobase pairs.

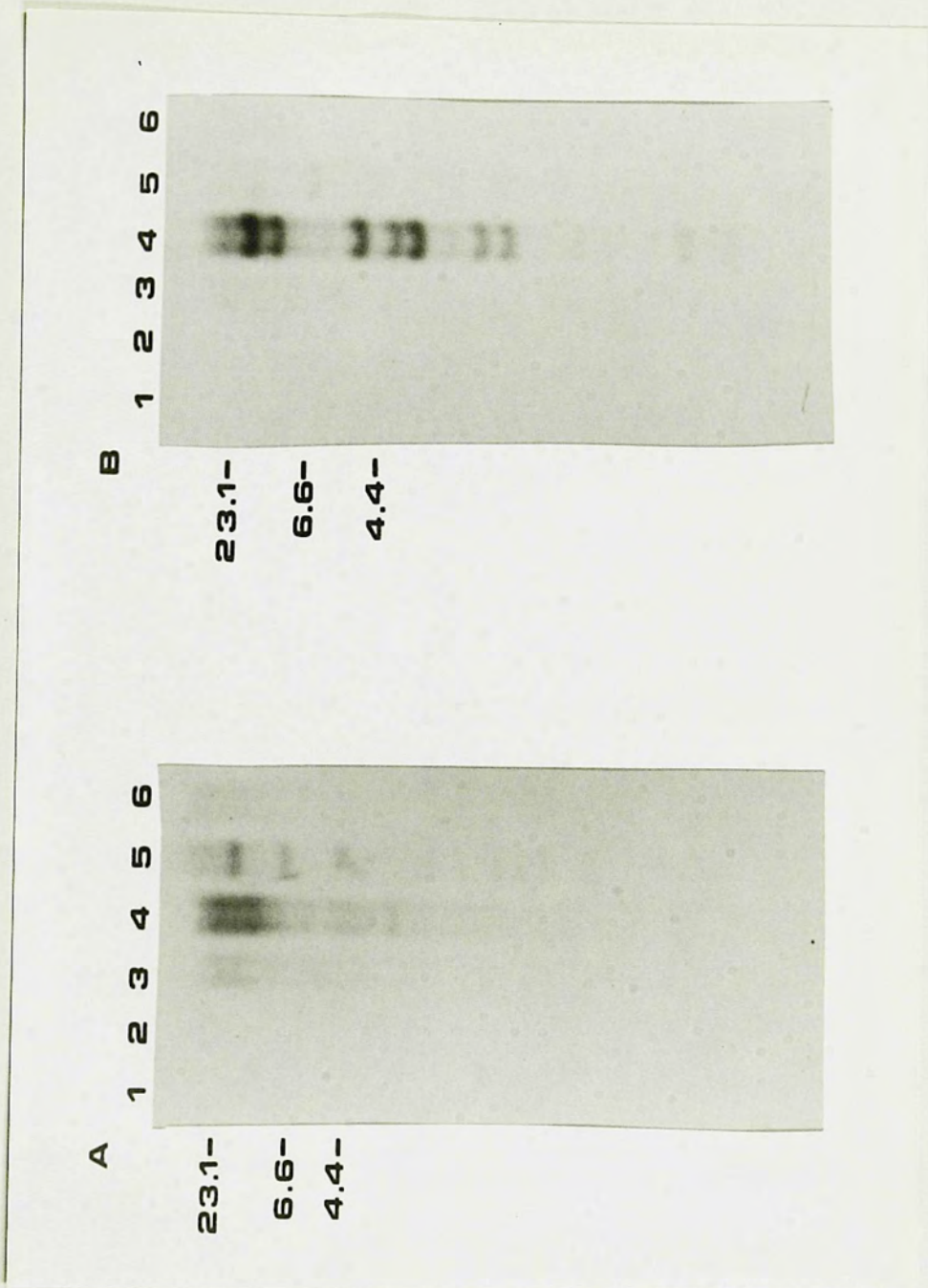
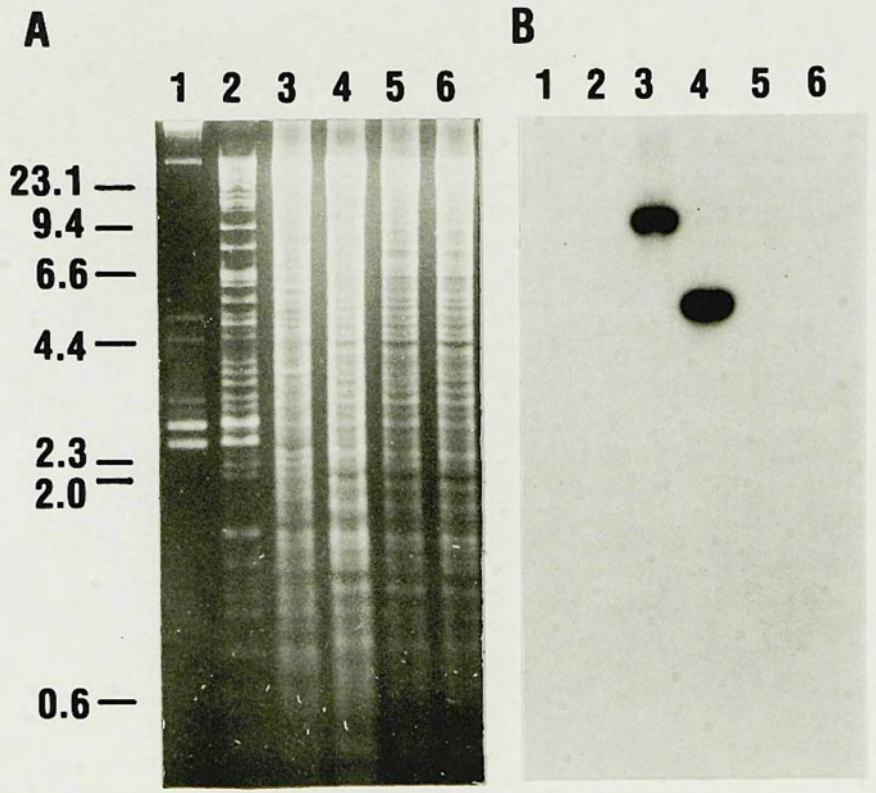

A
1 2 3 4 5 6
23.1-
6.6-
4.4-
B
1 2 3 4 5 6
23.1-
6.6-
4.4-

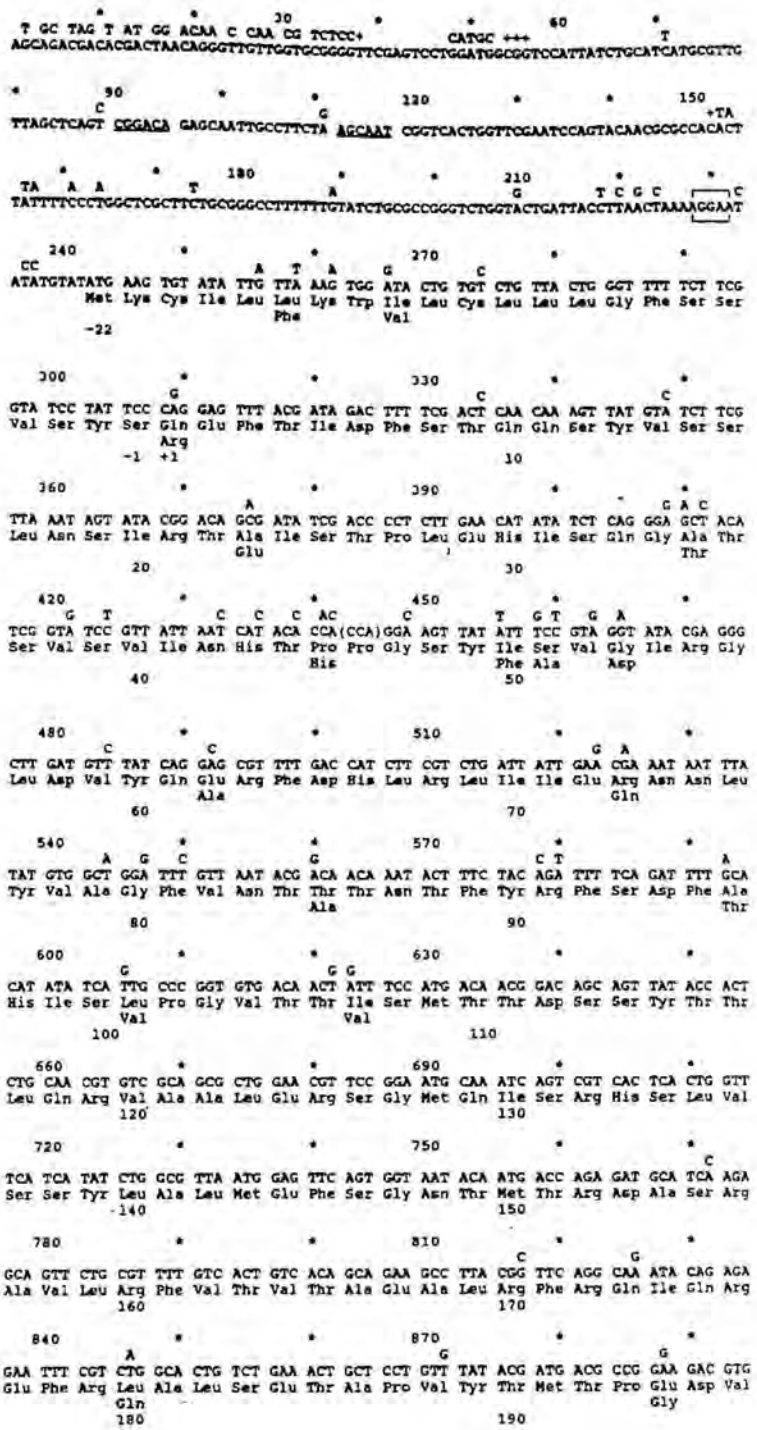
Figure 12. Southern blot analysis of cesium chloride purified plasmid DNA and EcoRI-digested plasmid and whole cell DNA samples. Panel A shows agarose gel electrophoresis of the DNA samples after ethidium bromide staining. Panel B shows the autoradiograph of the Southern blot of the gel in panel A after hybridization with the ^{32}P-labeled 1.06 kb EcoRV to PstI probe isolated from pDLW5. Hybridization conditions allowed for 20% base pair mismatch. The autoradiograph represents a 24 hour exposure in the presence of an intensifying screen (Eastman Kodak, Co., Rochester, N.Y.). Lanes 1, cesium chloride purified plasmid preparation from E. coli S1191; lanes 2, cesium chloride purified plasmid preparation from E. coli S1191 digested to completion with EcoR1; lanes 3-6, whole cell DNA isolated from E. coli S1191 (lanes 3), E. coli C600(933W) (lanes 4), E. coli C600(933J) (lanes 5) and E. coli C600 (lanes 6). Numbers on the left indicate sizes in kilobase pairs.



Nucleotide sequence analysis and comparison of slt genes. The nucleotide sequence of the slt-IIv genes was determined by the strategy shown in Figure 5. In Figure 13, the nucleotide and deduced amino acid sequences for slt-IIvA and slt-IIvB are compared to the reported nucleotide and deduced amino acid sequences for slt-IIA and slt-IIB (Jackson et al., 1987a). To align the open reading frames for the A subunit structural genes of SLT-IIv and SLT-II, an additional codon and the corresponding amino acid were inserted after nucleotide 442 of slt-IIvA. An untranslated space of 15 nucleotides separated slt-IIvA from slt-IIvB, whereas the intergenic space that separates slt-IIA from slt-IIB is 14 nucleotides (Jackson et al., 1987a). In the stx/slt-I operons the untranslated space between the A and B subunit genes is 12 nucleotides (Strockbine et al., 1988; Jackson et al., 1987; DeGrandis et al., 1987; Calderwood et al., 1987). Therefore, the A and B subunit genes of the slt-IIv and stx/slt-I operons are translated in the same reading frame while the A and B subunit genes of the slt-II operon are translated in different reading frames (Jackson et al., 1987a).

Presumed E. coli signal peptidase I cleavage sites (Oliver, 1985) were identified for the SLT-IIv A polypeptide and the SLT-IIv B polypeptide between amino acid residues -1 and +1 in Figure 13. The lengths of these putative signal peptides, 22 residues and 19 residues for the A and B subunits, respectively, are the same as the putative signal peptides for the SLT-II A and B polypeptide genes. The lengths of the coding sequences and deduced

Figure 13. Nucleotide sequence of the slt-IIvA and slt-IIvB structural genes. The slt-IIv nucleotide sequence is numbered above each line and the deduced SLT-IIv amino acid sequence is numbered below each line. The sequence is compared to the published slt-II sequence (Jackson et al., 1987a) from nucleotides 1 to 1667. The nucleotide sequence of slt-II is shown above the slt-IIv sequence only where the sequences are different and differences in the amino acids are shown below. The symbol (+) above nucleotides 56, 57, and 1200 indicates nucleotides that were present in slt-IIv but absent from slt-II. The 3 nucleotides enclosed by parentheses at positions 443-445 indicate a codon that was present in the SLT-IIv A subunit sequence but not present in the SLT-II sequence. The open reading frame for the A subunit gene is between nucleotides 242 and 1199 with the N-terminal glutamine of the processed polypeptide depicted as +1. The open reading frame for the B subunit gene is between nucleotides 1214 and 1474 with the N-terminal alanine depicted as +1. The proposed promoter sequences (Rosenberg and Court, 1979) are underlined at nucleotides 90-95 (-35) and 112-117 (-10). Two potential ribosome binding sites (Shine and Dalgarno, 1974) are bracketed beginning at nucleotides 228 (5' to slt-IIA) and 1203 (5' to slt-IIvB). A putative transcription terminator (Rosenberg and Court, 1979) is overlined from nucleotide positions 1819 to 1839.




900
930
960
990
1020
1050
1080
1110
1140
1170
1200
1230
1260
1290
1320
1350
1380
1410
1440
1470
1500
1530
1560
1590
1620
1650
1680
1710
1770
1800
1830
1860
1890

amino acid sequences of the SLT-IIv A and B subunits as well as their molecular weights and predicted isoelectric points (pIs) are given in Table 9 and compared to the corresponding values for Shiga toxin/SLT-I and SLT-II. The processed SLT-IIv A subunit consisted of 297 amino acids and was four amino acid residues longer than the Shiga/SLT-I A subunit and one amino acid residue longer than the SLT-II A subunit. The processed SLT-IIv B subunit consisted of 68 amino acids and was one amino acid shorter than the Shiga/SLT-I B subunit and two residues shorter than the SLT-II B subunit. The A subunit of SLT-IIv had a calculated molecular weight of 33,050 and the B subunit of SLT-IIv had a calculated molecular weight of 7,565. The A subunit of SLT-IIv (pI=8.7) was more acidic than the A subunits of Shiga toxin/SLT-I (pI=11.1) and SLT-II (pI=9.8). In contrast, the B subunit of SLT-IIv (pI=10.2) was considerably more basic than the B subunits of Shiga/SLT-I (pI=5.9) and SLT-II (pI=5.4). The basic pI of the SLT-IIv B subunit reflected the differences in the number of charged amino acids (at neutral pH). For example, two acidic residues of the SLT-II B subunit, aspartic acid and glutamic acid at positions 17 and 64, were substituted by corresponding neutral amino acids, asparagine and glutamine in SLT-IIv. Other differences between the amino acids of the B subunits of SLT-II and SLT-IIv that affected charge included: position 24, aspartic acid for SLT-II and serine for SLT-IIv; position 57, glutamic acid for SLT-II and serine for SLT-IIv; and, position 66, glutamine for SLT-II and lysine for SLT-IIv.

The nucleotide and predicted amino acid sequence homologies of

Table 9. Comparisons of the processed SLT-IIv, Shiga toxin/SLT-I and SLT-II subunit

	A Subunit			B Subunit		
	SLT-IIv	Shiga/ SLT-I[a]	SLT-II	SLT-IIv	Shiga/ SLT-I[a]	SLT-II
Nucleotides	891	879	888	204	207	210
Amino Acid Residues	297	293	296	68	69	70
Molecular Weight	33,050	32,225/ 32,211	33,135	7,565	7,690	7,817
Isoelectric Point	8.7	11.1	9.8	10.2	5.9	5.4

[a]The Shiga toxin and SLT-I A subunits have one amino acid difference, a threonine at position 45 in Shiga toxin and a serine at the corresponding position in SLT-I. This difference is reflected by a slight difference in the molecular weights of the A subunits.

the processed forms of the A and B subunits of SLT-IIv are compared with SLT-II and Shiga toxin/SLT-I in Table 10. The nucleotide sequences of the genes for the A subunits of SLT-IIv and SLT-II were highly homologous (94%) while the nucleotide sequences of the genes for the B subunits were less homologous (79%). The nucleotide sequences of the genes for the A and B subunits of SLTIIv and Shiga toxin/SLT-I were only 60% and 64% homologous, respectively. Likewise, the deduced amino acid sequences of the A subunits of SLT-IIv and SLT-II were highly homologous (93%) while the B subunits were less homologous (84%). The deduced amino acid sequences of the A and B subunits of SLT-IIv and Shiga toxin/SLT-I revealed only 56% and 61% homologies, respectively. As determined in a previous study (Jackson et al., 1987), the deduced amino acid sequences of the processed A and B subunits of SLT-II and Shiga toxin (SLT-I) have 55% and 57% homology, respectively.

A three way comparison among SLT-IIv, SLT-II, and Shiga/SLT-I revealed that 5 of the 18 amino acids that differed between the processed A subunits of SLT-IIv and SLT-II at residues 23,73,85,194, and 291 (Figure 13) were identical in the A subunits of SLT-IIv and Shiga toxin/SLT-I. Similarly, 2 of the 11 amino acids that differed between the processed B subunits of SLT-IIv and SLT-II at residues 31 and 54 (Figure 13) were identical in SLT-IIv and Shiga toxin/SLT-I. Despite these differences, the number and locations of the cysteine residues in the processed SLT-IIv A and B subunits (residues 241 and 260 in the SLT-IIv A subunit and residues 3 and 56 in the SLT-IIv B subunit) were identical in the corresponding subunits of SLT-II and

Table 10. Nucleotide and deduced amino acid sequence homologies of the processed SLT-IIv subunits with the Shiga toxin/SLT-I and SLT-II subunits.

	Nucleotide sequence homology with SLT-IIv (%)		Deduced amino acid sequence homology with SLT-IIv(%)	
Toxin	A subunit	B subunit	A subunit	B subunit
SLT-II	94	79	93	84
Shiga toxin/ SLT-I	60	64	56	61

Shiga toxin/SLT-I. As predicted due to the high degree of sequence homology, the hydropathy plots for the SLT-IIv and SLT-II polypeptides were nearly identical (data not shown).

A putative promoter sequence (Rosenberg and Court, 1979) for the slt-IIv operon was identified upstream to the A subunit coding region (at nucleotides 90 and 112, underlined in Figure 13). This promoter sequence was identical to the promoter sequence identified for the slt-II operon by primer extension analysis, S1 nuclease analysis, and deletion mutagenesis (Sung, L., unpublished observations). A putative ribosome binding site (Shine and Dalgarno, 1974) was located immediately preceding the slt-IIv A gene at nucleotides 218-222. A second putative ribosome binding site was located within the untranslated space separating slt-IIv A from slt-IIv B, at nucleotides 1203-1207, (bracketed in Figure 13). Putative ribosome binding sites have also been identified within the untranslated spaces between the A and B subunit genes of SLT-I/Shiga toxin and SLT-II (Calderwood et al., 1987; DeGrandis et al., 1987; Jackson et al., 1987, 1987a; Strockbine et al., 1988). A putative transcriptional terminator structure (Rosenberg and Court, 1979) was identified 3' to the stop codon of slt-IIvB (from nucleotide 1819 to 1839, overlined in Figure 13).

Nucleotide sequences flanking the slt-IIv operon were compared to the corresponding flanking sequences in slt-II. Upstream sequences between nucleotides 58 and 241 (Figure 13) of slt-IIv and slt-II were 89% homologous. In contrast, sequences 3'to the slt-IIv B and slt-II B terminator codons (between nucleotides 1483

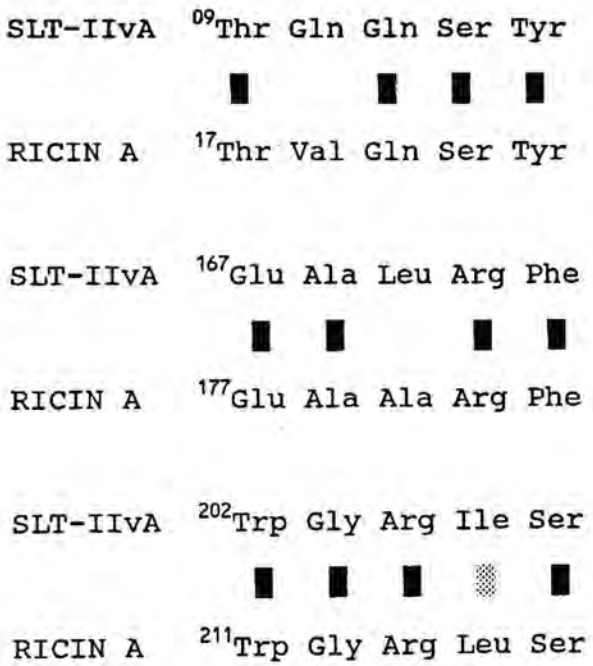
and 1667) were only 19% homologous. The slt-II B nucleotide sequence beyond nucleotide 1667 of slt-IIv B has not been determined.

Homology with ricin. Shiga toxin/SLT-I, SLT-II and the plant lectin ricin are ribosomal RNA N-glycosidases (Endo et al., 1987, 1988). In previous studies, regions in the A subunit of ricin have been found to share significant homology with the A subunit of SLT-I (Calderwood et al., 1987; DeGrandis et al., 1987). To assess the degree of relatedness between the SLT-IIv A subunit and the ricin A chain (Lamb et al., 1985), polypeptides were compared using the MicroGenie alignment program. The overall homology of the processed forms of the SLT-IIv A subunit and the ricin A chain was 19%. The homology function was then used to select regions within the aligned sequences using a window of four common residues with at least 75% or greater homology. Three such regions in the SLT-IIv A subunit were identified that shared between 75-80% homology (Figure 14). These same regions of homology were also highly conserved in the deduced amino acid sequences of the Shiga toxin/SLT-I and SLT-II A subunits. One of these regions included glutamic acid residue 167. Hovde et al. (1988) recently provided evidence that the corresponding residue in the SLT-I A subunit is critical for enzymatic activity.

III. Receptor binding, cell specificity, and extracellular localization studies.

Receptor-binding studies. A receptor-analogue ELISA was used to determine if SLT-IIv binds to the same receptor as does Shiga

Figure 14. Regions of homology between the deduced amino acid sequences of SLT-IIv A subunit and the ricin A chain. Three regions of 75% or greater homology between the deduced amino acid sequence of SLT-IIv A subunit and the ricin A chain (using a window of four residues) were selected using the MicroGenie homology program after the two sequences were aligned using the MicroGenie alignment program. The N-terminal residue of each sequence is numbered. The symbol (▉) indicates identity, whereas the symbol (▒) indicates a conservative amino acid substitution.


SLT-IIvA 09Thr Gln Gln Ser Tyr
RICIN A 17Thr Val Gln Ser Tyr
SLT-IIvA 167Glu Ala Leu Arg Phe
RICIN A 177Glu Ala Ala Arg Phe
SLT-IIvA 202Trp Gly Arg Ile Ser
RICIN A 211Trp Gly Arg Leu Ser

toxin/SLT-I (Jacewicz, et al., 1986; Lindberg et al., 1986, 1987; Lingwood et al., 1987) and SLT-II (Waddell et al., 1988; J. E. Brown, R. J. Neill, A. D. O'Brien, and A. A. Lindberg. Abstr. Int. Symp. Workshop on Verocytotoxin-producing E. coli Infect. 1987. STF-3). Sonic lysate preparations of SLT-IIv and SLT-II (concentrated with ammonium sulfate precipitation and then diluted to $1x10^4$-$1x10^6$ CD_{50}/ml) were tested for binding to Gal-Gal-BSA in an ELISA. All dilutions of the SLT-II preparations bound at least 100-fold more to wells coated with Gal-Gal-BSA than to wells incubated with PBS alone. By contrast, no significant increase in SLT-IIv binding was detected in wells coated with Gal-Gal-BSA when compared to wells incubated with PBS alone (data not shown). These results suggest that SLT-IIv does not bind a Gb_3-receptor analogue.

Subunit Complementation. E. coli HB101 was co-transformed with plasmids carrying the individual A and B subunit genes of SLT-I, SLT-II, and SLT-IIv (Figure 8) to produce hybrid cytotoxins in vivo. Sonic lysates and culture supernatants were tested for cytotoxicity on Vero and HeLa cells to determine the levels of cell-associated and extracellular cytotoxin produced by the co-transformants (Table 11). E. coli HB101 co-transformed with individual subclones encoding homologous subunits always produced levels of cytotoxin equivalent to or higher than the levels produced by the original plasmids. This indicated that all of the individual subclones produced intact A and B subunits that can complement in vivo to give active holotoxin molecules. E. coli HB101 co-transformed with pDLW102 and pJN26 (slt-II A and slt-I B)

Table 11. Complementation studies: Cytotoxicity on Vero and HeLa cells from E. coli HB101 producing hybrid toxins.

Toxin		Cytotoxicity for Vero cells			Cytotoxicity for HeLa cells		
A subunit	B subunit	Cell associated[a]	Extracellular[a]	% Extracellular[b]	Cell associated	Extracellular	% Extracellular
I	I	7.4	5.6	1	7.4	5.6	1
I	II	ND[c]	ND	ND	ND	ND	ND
I	IIv	ND	ND	ND	ND	ND	ND
II	I	6.4	3.6	<1	6.4	3.6	<1
II	II	6.4	5.6	14	6.4	5.6	14
II	IIv	3.4	3.6	62	ND	ND	ND
IIv	I	6.4	3.6	<1	5.4	3.6	1.4
IIv	II	6.4	5.6	14	6.4	5.6	14
IIv	IIv	6.4	7.6	94	ND	ND	ND

[a] Cell associated, $\log_{10}(CD_{50}$/pellet); extracellular, $\log_{10}(CD_{50}$/40 ml of supernatant).

[b] % extracellular, [extracellular/(cell associated plus extracellular)]x100.

[c] No cytotoxicity detected above the levels produced by E. coli HB101.

produced approximately $2.5x10^6$ total CD_{50} (cell-associated plus extracellular) of both Vero and HeLa cell cytotoxin. E. coli co-transformed with pDLW102 and pDLW105 (slt-II A and slt-IIv B) produced a hybrid toxin with approximately $6.5x10^3$ total CD_{50} for Vero cells only. This hybrid toxin with the SLT-IIv B subunit was not cytotoxic for HeLa cells. E. coli co-transformed with pDLW104 and pJN26 (slt-IIv A and slt-I B) or pDLW103 (slt-IIv A and slt-II B) produced approximately $2.5x10^6$ total CD_{50} for both Vero and HeLa cells. Using 16-fold concentrations of sonic lysates, no cytotoxicity above the levels produced by E. coli HB101 alone was detected from E. coli co-transformed with pDLW101 and pDLW103 (slt-I A and slt-II B) or pDLW105 (slt-I A and slt-IIv B). This suggested that either the Shiga toxin/SLT-I A subunit could not combine with a heterologous B subunit or that the hybrid cytotoxin formed was biologically inactive.

As revealed in Table 11, cytotoxins containing the SLT-I or SLT-II B subunits were localized predominantly in the cell-associated fractions (>99% and 86%, respectively), whereas cytotoxins containing SLT-IIv B subunits were 94% localized to the extracellular milieu. No cytotoxicity was detected above the levels produced by E. coli HB101 from transformants carrying plasmids expressing the individual A or B subunits (data not shown).

Operon fusions. Because of the possibility that the copy number of the individual plasmids expressing A or B subunits might be different, hybrid cytotoxins produced by operon fusions were

studied to confirm the complementation data. Unlike the subunit complementation analyses, both the A and B subunit genes of the fused hybrid operons were transcriptionally regulated by the naturally occurring promoter 5' to the A subunit gene. Sonic lysates and culture supernatants of E. coli HB101 transformed with pMJ153 (stx), pMJ330 (slt-II), pDLW5.321 (slt-IIv) or the six operon fusions were tested on HeLa and Vero cells to determine the levels of cell-associated and extracellular cytotoxin (Table 12). The cytotoxicity profiles of the hybrid toxins produced by the operon fusions were similar to those observed in subunit complementation studies. As shown in Table 3, any cytotoxins containing SLT-I B or SLT-II B were localized predominantly in the cell-associated fractions (>99% and 86%, respectively), whereas cytotoxins containing SLT-IIv B subunits were 94% localized to the extracellular milieu. The hybrid cytotoxins containing the Shiga toxin B subunit were neutralized by polyclonal anti-Shiga toxin serum, and the hybrid cytotoxins containing the A or B subunits of SLT-II or SLT-IIv were neutralized by polyclonal anti-SLT-II serum (data not shown). Therefore, the cytotoxic specificity and localization of hybrid cytotoxins corresponded to the source of the B subunit.

Analysis of hybrid molecules with reduced cytotoxicity. Dot blot and immunoprecipitation analyses were performed by L. P. Perera on cell extracts of the transformants which produced reduced levels of hybrid cytotoxins (Shiga/SLT-I A subunit gene with SLT-II or SLT-IIv B subunit gene) to determine whether the B subunit was

Table 12. Operon fusion studies: Cytotoxicity on Vero and HeLa cells from E. coli HB101 producing hybrid toxins.

Plasmid	Toxin		Cytotoxicity for Vero cells			Cytotoxicity for HeLa cells		
	A subunit	B subunit	Cell associated[a]	Extracellular[a]	% Extracellular[b]	Cell associated	Extracellular	% Extracellular
pMJ153	Shiga	Shiga	7.4	5.6	2	7.4	5.6	2
pFUS3	Shiga	II	ND[c]	ND	ND	ND	ND	ND
pFUS5	Shiga	IIv	ND	ND	ND	ND	ND	ND
pFUS4	II	Shiga	5.4	3.6	2	5.4	3.6	2
pMJ330	II	II	6.4	5.6	14	5.4	4.6	14
pFUS2	II	IIv	4.4	4.6	62	ND	ND	<1
pFUS6	IIv	Shiga	5.4	3.6	2	5.4	ND	<1
pFUS1	IIv	II	4.4	3.6	14	4.4	ND	<1
pDLW5.321	IIv	IIv	4.4	4.6	62	ND	ND	ND

[a] Cell associated, $\log_{10}(CD_{50}/\text{pellet})$; extracellular, $\log_{10}(CD_{50}/40$ ml of supernatant).

[b] % extracellular, [extracellular/(cell associated plus extracellular)]x100.

[c] No cytotoxicity detected above the levels produced by E. coli HB101

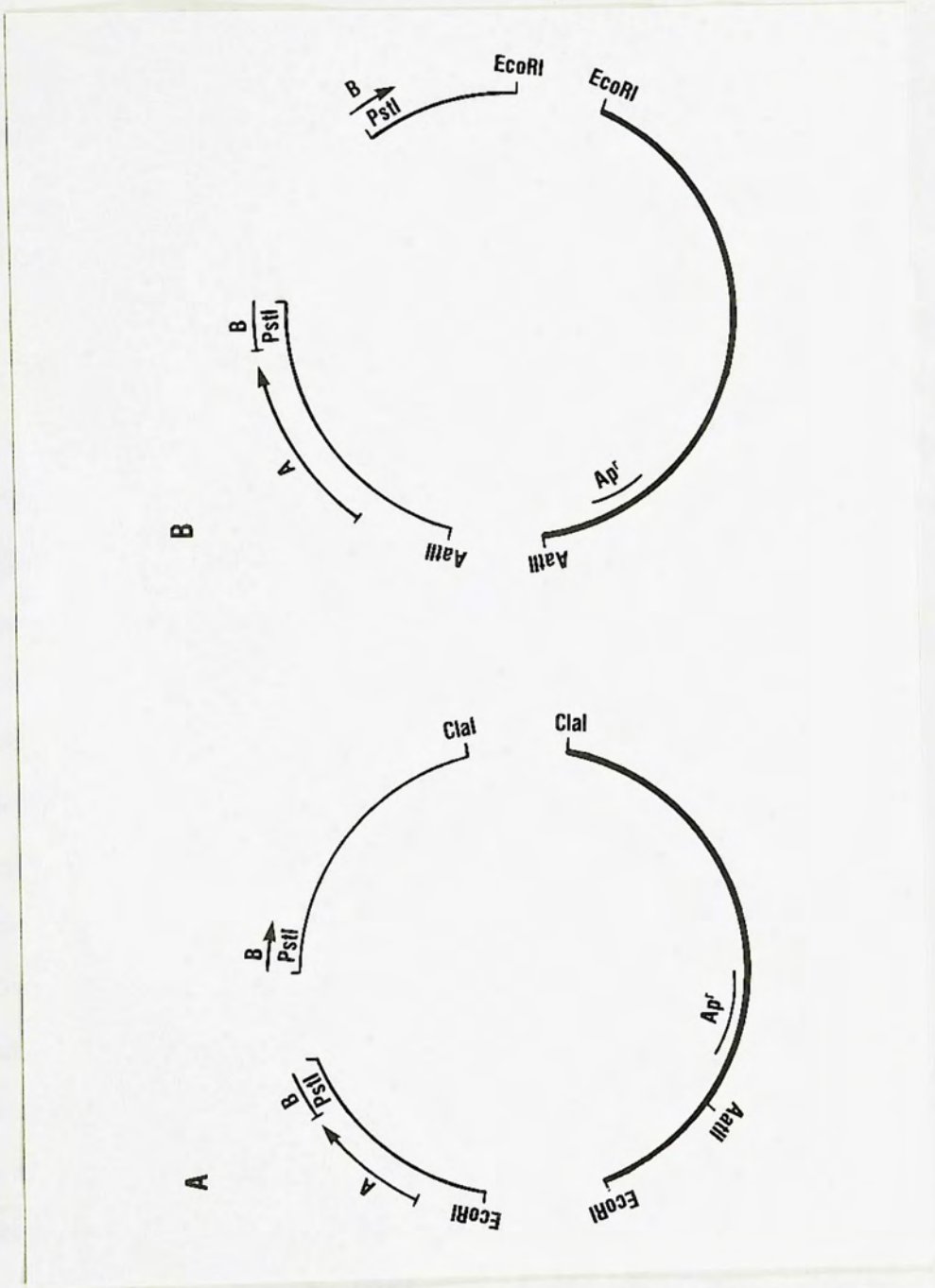
produced and if it assembled with the Shiga toxin/SLT-I A subunit. E. coli transformed with individual subunit plasmids or operon fusions produced immunoreactive B subunits as assessed by dot blot analysis. Moreover, as revealed by immunoprecipitation studies, the Shiga toxin/SLT-I A subunit gene was expressed and the A subunit was assembled with a heterologous B subunit; polypeptides corresponding in molecular weights to the processed Shiga toxin/SLT-I A subunit (M_r, 32,000) and the processed SLT-II or SLT-IIv B subunit (M_r,7000) were immunoprecipitated by the SLT-I A and SLT-II B subunit-specific monoclonal antibodies (results not shown). The results in Tables 11 and 12 represent a 16-fold concentration of toxin in sonic lysates. When the sonic lysates of E. coli transformed with pDLW101 and pDLW103 or pDLW105, pFUS3, or pFUS5 were concentrated 250-fold by ammonium sulfate precipitation for the dot blot and immunoprecipitation studies, low levels of cytotoxin were detected. These findings indicate that the Shiga toxin/SLT-I A subunit can combine with a heterologous B subunit to form very low levels of biologically active cytotoxin.

SLT-IIv B subunit mutagenesis. A three way comparison among SLT-IIv, SLT-II, and Shiga toxin/SLT-I revealed that 3 of the 11 amino acids that differed between the processed B subunits of SLT-IIv and SLT-II at residues 17, 52, and 64 (Figure 13) were identical in the corresponding subunits of Shiga toxin/SLT-I and SLT-II. In addition, these three residues represented differences in charges (Asn_{17} in SLT-IIv is an Asp in Shiga toxin/SLT-I and SLT-II; Ile_{52} in SLT-IIv is a Lys in Shiga toxin/SLT-I and SLT-II; and, Gln_{64} in

SLT-IIv is a Glu in Shiga toxin/SLT-I and SLT-II). To assess the roles of these three residues in the different binding specificity or extracellular localization of SLT-IIv as compared to Shiga toxin/SLT-I or SLT-II, the residues were changed using oligonucleotide-directed site-specific mutagenesis to the residue found in the same positions in Shiga toxin/SLT-I and SLT-II. The 1.5 kb EcoRI to PstI fragment in pDLW5.3 (Figure 6) was cloned into M13mp19 to generate the template used for the mutations in pBMUTV1 and pBMUTV3, and the 0.5 kb PstI to EcoRI fragment in pDLW5.5 was cloned into M13mp18 to generate the template used for mutation in pBMUTV2. Once the desired mutation was stably incorporated into the DNA, the corresponding fragment was electroeluted and religated to the remainder of the operon into plasmid vector pBR329 (Figure 15). The nucleotide sequences of the 3' end of the A subunit through the mutation in the B subunit were determined to verify that the reconstructed operon was properly aligned. The EcoR1 to PstI fragment carrying either the mutation from pBMUTV1 or pBMUTV3 was ligated to the PstI to EcoRI fragment from pBMUTV2 into pBR329 to create toxins which carried two amino acid changes (pBMUTV1.2 and pBMUTV2.3; Figure 15).

To assess the effects of these mutations on cytotoxicity levels, specificity, and cellular localization, E. coli HB101 was transformed with plasmids carrying the B subunit gene mutation(s) and intact A subunit gene to produce variant cytotoxins in vivo. Sonic lysates and culture supernatants of the transformants were tested for cytotoxicity on Vero and HeLa cells to determine the

Figure 15. Strategies for constructing the slt-IIv operon with B subunit gene mutations. Panel A. Restriction fragments used for reconstructing pBMUT1 and pBMUT3. Panel B. Restriction fragments used for reconstructing pBMUT2, pBMUT1.2, and pBMUT2.3. The SLT-IIv structural genes, slt-IIv A and slt-IIv B, are oriented above the restriction maps. Vector DNA (pBR329) is indicated by a bold line.


B
EcoRI
EcoRI
B
PstI
B
PstI
A
Ap^r
AatII
AatII
A
ClaI
ClaI
B
PstI
B
PstI
Ap^r
A
AatII
EcoRI
EcoRI

levels of cell-associated and extracellular cytotoxin (Table 13). None of the mutant toxins was cytotoxic for HeLa cells. As seen in Table 13, cytotoxins containing the Gln_{64} to Glu mutation were localized predominantly in the cell-associated fractions (86 to 99%) whereas the wild type and other mutant cytotoxins were 61 to 100% localized to the extracellular milieu.

IV. Creation of non-toxinogenic E. coli S1191.

Plasmid construction. As a first step in the creation of a non-toxinogenic variant of ED-causing E. coli S1191, a series of different plasmids carrying the mutant, non-toxic slt-IIv gene were constructed (see Table 4 and "Materials and Methods" section).

Transformation, conjugation, and transduction of strain S1191. The second step in construction of an SLT-IIv-negative derivative of S1191 was to introduce these constructs into S1191. Numerous attempts were made to introduce the above constructs or control plasmids (the suicide vector pGP704 or the multicopy plasmid vectors pBR329 or pACYC184) into ED-causing E. coli strain S1191. No transformants were isolated using Hanahan's calcium chloride transformation procedure (Hanahan, 1983). When the bacteria were treated with calcium chloride and 15% DMSO, 10 transformants of pBR329 and 1 transformant of pACYC184 per 10 μg DNA were detected. In one trial out of four, electroporation of E. coli S1191 resulted in 5 transformants of pDLW5.104, 6 transformants of pDLW5.1041, and 2 transformants of pDLW5.1042 per 10 μg of purified DNA. Attempts to co-transform these transformants with a second plasmid (as a means to eliminate the first plasmid; see next section) were

Table 13. B subunit mutagenesis studies: Cytotoxicity on Vero cells from E. coli HB101 producing mutant toxins.

Plasmid	Mutation	Cytotoxicity		
		Cell associated[a]	Extracellular[b]	% Extracellular
pDLW5	none	5.4	5.6	62
pBMUTV1	Asn_{17} to Asp	5.4	5.6	62
pBMUTV2	Gln_{64} to Glu	4.4	3.6	14
pBMUTV3	Ile_{52} to Lys	2.4	4.6	99
pBMUTV1.2	Asn_{17} to Asp Gln_{64} to Glu	3.4	ND	<1
pBMUTV2.3	Gln_{64} to Glu Ile_{52} to Lys	3.4	ND	<1

[a] Cell associated, $\log_{10}(CD_{50}$/pellet); extracellular, $\log_{10}(CD_{50}$/40 ml of supernatant).

[b] % extracellular, [extracellular/(cell associated plus extracellular)]x100.

[c] No cytotoxicity detected above the levels produced by E. coli HB101.

unsuccessful. When plasmids isolated from transformants of S1191 were used to transform wild type S1191, the transformation efficiency did not increase. This finding suggested that the low transformation efficiency of S1191 was not due to the degradation of incoming plasmids by restriction enzymes.

No E. coli S1191 transconjugants or plaques were detected when S1191 was used as a recipient in conjugation experiments or a host in transduction experiments with bacteriophage lambda, M13 or P1. This is in contrast to the results with the E. coli HB101 (used as a control) which did act as a recipient in conjugation experiments or as a host in transduction experiments with the same bacteriophage. This series of experiments indicated that the only way to introduce DNA into E. coli S1191 was by transformation with DMSO or by electroporation. However, the frequency of transformation was very low.

Attempts to force recombination of wild type slt-IIv gene with mutant gene. The third step in the construction of a nontoxinogenic derivative of E. coli S1191 was to force the recombination of the wild type slt-IIv A subunit gene with the mutant gene and then eliminate the vector carrying the cloned genes. Several approaches were attempted to achieve this two-step process. First, I tried to use the suicide vector pGP704, which is a derivative of pJM703.1 (Miller and Mekalanos, 1988). This vector will not replicate in any strain that does not carry the pir gene, and indeed, E. coli S1191 did not support the replication of this suicide vector (e.g., no transformants of pGP704 without an

insert were isolated). If mutant slt-IIv toxin genes are cloned into pGP704, any transformants should actually be recombinants (e.g., the mutant DNA has integrated into an homologous region of the chromosome, which in this case is the wild type slt-IIv gene). Unfortunately, no transformants of pDLW5.1043 (mutant slt-IIv gene in pGP704) were obtained. Thus, attempts to use this suicide vector as a method for gene replacement were not successful.

The other two methods used to attempt the replacement of wild type slt-IIv genes with the insertionally inactivated slt-IIv genes took into account the relative resistance of E. coli S1191 to the introduction of foreign DNA (see preceding section). Method two involved successive subculturing without antibiotic pressure of E. coli S1191 transformed with pDLW5.104 (Ap^r) or pDLW5.1041 (Km^r/Ap^r). The idea was to promote the loss of each plasmid and the replacement of wild type genes with mutant slt-IIv genes. These transformants were subcultured up to 5 times without antibiotic selection. Some Ap^s but no Km^r/Ap^s transformants were isolated. All of the Ap^s colonies were Vero cell cytotoxic which indicated that the wild type gene had not been replaced by the mutant gene.

The third method attempted for gene replacement was ampicillin/D-cycloserine cycling. The principle of this technique is that cells which harbor a plasmid vector that expresses Cm^r are killed when cultured in the presence of ampicillin and/or D-cycloserine, whereas Cm^s cells (those that have lost the plasmid) are not killed (chloramphenicol is a bacteriostatic, not a bacteriocidal, agent). The S1191 transformants that contained

pDLW5.1042 (Km^r, Cm^r) were subcultured five times without antibiotic selection, grown to mid-logarithmic phase in the presence of chloramphenicol, and then actively dividing cells were lysed with high concentrations of D-cycloserine and ampicillin. Even after two cycles of this procedure, all of the remaining cells were Cm^r which indicated that S1191 is somewhat resistant to D-cycloserine and ampicillin, and thus these antibiotics were not useful for lysing E. coli S1191. Thus, I was unable to create a nontoxinogenic variant of E. coli S1191 by any of the three standard methods for gene replacement described above. Nonetheless, a number of subclones were constructed which may be useful in the creation of such a nontoxinogenic strain in future studies.

Discussion

I. Regulation studies.

One of the goals of this dissertation project was to analyze the organization and regulation of the SLT-I operon in E. coli and compare it to the Shiga toxin operon of S. dysenteriae 1. Subsequently, the effect of iron on SLT-II and SLT-IIv production was assessed. Chronologically, the organization of the SLT-I operon was examined first. At that time, only SLT-I had been cloned but none of the toxin genes had been sequenced. However, it was known that Shiga toxin consisted of a single A subunit noncovalently linked to 5 copies of the B subunit (Donohue-Rolfe et al., 1984). Because purified SLT-I appeared to be biochemically and immunologically indistinguishable from Shiga toxin (O'Brien and LaVeck, 1982), it was presumed that the A:B stoichiometry was the same for SLT-I.

Two possible mechanisms for the production of proportionally more Shiga toxin/SLT-I B subunits as compared to A subunits were proposed. The first possibility, which was based on studies with the cholera toxin operon (Mekalanos et al., 1983), was that the stx/slt-I A and B subunit genes are translated independently from a polycistronic messenger RNA but that translation of the B subunit is more efficient. The second possible mechanism is that a second promoter exists for the stx/slt-I B genes. These two explanations are not mutually exclusive.

The data in support of independent translation of the A and B subunits of Shiga toxin/SLT-I from a single polycistronic

messenger RNA are from the analyses of the nucleotide sequences that were accomplished by Jackson et al. (1987) and Strockbine et al. (1988). Each operon has a conserved ribosome binding sequence both 5' to the A subunit genes and in the untranslated space between the A and B subunit genes. Moreover, a subsequent comparison of these operons with the slt-II (Jackson et al., 1987a) and slt-IIv operons (cloned and sequenced in this project; Weinstein et al., 1988; Gyles et al., 1988) demonstrated that the organization of the operons of all the toxin family members is similar.

The results on the location of the slt-I A and slt-I B genes from the mini-Mu lac operon studies described herein are consistent with the nucleotide sequence data obtained by Jackson et al. (1987). Furthermore, mini-Mu lac operon fusion analysis confirmed that the direction of transcription of the genes was as originally proposed by Newland et al. (1985) and provided evidence to support the existence of an independent promoter for slt-I B. This second promoter may enhance the transcription of the B subunit gene. S1 nuclease protection studies by DeGrandis et al. (1987) suggest, but do not definitively prove, that the second promoter functions in vivo.

In the second part of the regulation studies, the effect of iron on SLT-I production was assessed. I observed that iron suppressed cytotoxin synthesis by E. coli C600(933J) and, as expected from the studies of others (Dubos and Geiger, 1946; van Heyningen and Gladstone, 1953; McIver et al., 1975; O'Brien and

LaVeck, 1982), iron also affected Shiga toxin synthesis by S. dysenteriae 1 grown at 37°C (Weinstein et al., 1987). In contrast, iron had no demonstrable effect on β-galactosidase activity in slt-I operon fusions (Table 2), nor did it alter cytotoxin production by E. coli strains transformed with pJN25 (slt-I operon cloned in pBR329) and cultured at 37°C.

Other investigators also assessed the effect of iron on SLT-I production. DeGrandis et al. (1987) demonstrated that the transcription of the slt-I gene was increased in iron-depleted media. Calderwood and Mekalanos (1987a) constructed Tn::PhoA fusions with the slt-I A subunit gene and were able to demonstrate the iron regulation of the promoter for slt-I A by measuring alkaline phosphatase levels. One possible explanation to resolve the discrepancies between the findings of Calderwood and Mekalanos (1987a) and my inability to demonstrate the iron regulation of β-galactosidase in slt-I operon fusions is as follows. The number of copies of the mini-Mu lac fusions with slt-I per cell may have been sufficiently high to titrate out all the available fur gene product. Since only one copy of fur exists in E. coli, there may not be enough fur gene product in the cell to regulate multiple copies of the toxin gene. Conversely, the operon fusions made by Calderwood and Mekalanos (1987a) may have been lower copy number than the operon fusions I constructed. Hence, the amount of endogenous fur gene product was apparently sufficient to regulate the phoA-slt-I A fusions.

Other phage-encoded toxin genes are known to be regulated by

iron. For example, expression of the diphtheria toxin operon of the β phage of Corynebacterium diphtheriae has been postulated to be negatively regulated by iron functioning as a corepressor in conjunction with a corynebacterial regulatory protein (Murphy et al.,1976). Betley et al. (1986) suggested a similar mechanism for the iron regulation of SLT-I synthesis involving the fur gene. In the model proposed by Betley et al. (1986), iron functions as a corepressor in conjunction with the fur gene product to bind putative operator sequences and inhibit transcription of slt-I. Analysis of the nucleotide sequence the slt-I operon revealed a putative consensus site for binding the fur gene product in the promoter regions (Calderwood et al., 1987, DeGrandis et al., 1987). The importance of the fur gene product was confirmed by Calderwood et al. (1987, 1988) who demonstrated that the slt-I operon was not regulated by iron in an E. coli containing a nonfunctional fur gene.

The slt-IIv and slt-II promoter sequences showed no consensus sites for binding to the fur gene product, and, as demonstrated in this report and by Weinstein et al. (1988), iron did not suppress SLT-IIv or SLT-II production. These results were confirmed by Sung et al. who demonstrated that the levels of SLT-II synthesis were not affected by the presence or absence of a functional fur gene (L. Sung, D. Weinstein, M. Jackson, and A. O'Brien, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1988, D-77 p. 84). Therefore, the regulation of synthesis of SLT-IIv and SLT-II differs from that of Shiga toxin/SLT-I.

In the third part of the regulation studies, the effect of temperature on Shiga toxin production by S. dysenteriae type 1 and SLT-I production by E. coli was examined. The experiments reported here and by Weinstein et al. (1987) demonstrated for the first time that production of Shiga toxin is not only regulated by iron but also by growth temperature. The mechanism of Shiga toxin temperature regulation is not known, but the preliminary data presented in this study indicate that the virR gene, which controls the temperature regulation of some virulence factors in Shigella spp. (Maurelli et al., 1984), is not involved. Because growth temperature did not affect the production of SLT-I in E. coli, S. dysenteriae 1 must possess a mechanism for the temperature control of Shiga toxin production that is not operative in SLT-I producing E. coli.

II. Cloning and sequencing of the SLT-II variant genes.

A second goal of this dissertation project was to clone the slt-IIv genes from the chromosome of ED-causing E. coli S1191 and determine the nucleotide sequences. The SLT-II variant produced by E. coli strains that cause edema disease of swine differs from other SLTs in that it is cytotoxic for Vero cells but not for HeLa cells. Because SLT-IIv is neutralized by polyclonal antisera specific for SLT-II and shares many of the biological characteristics of Shiga toxin and the other SLTs, Marques et al. (1987) speculated that differences in the binding subunit are responsible for the different cytoxic specificity. A comparison of the deduced amino acid sequences supports this hypothesis. The

deduced amino acid sequences revealed that the A subunits of the two toxins share considerable homology (94%), whereas the B subunits are less similar (84%). These amino acid differences are reflected in a marked difference in the pIs of the B subunits of these toxins [10.2 for the B subunit of SLT-IIv versus 5.4 for the B subunit of SLT-II (Table 9)]. This dramatic difference in pIs was the result of significant differences in the number of negatively charged amino acids in the B subunits of SLT-IIv and SLT-II (5 and 10, respectively).

Sequence comparisons between the A subunits of SLT-IIv and the A subunit of ricin identified three regions which were 75% or more homologous (Figure 14). The N-glycosidase activity of ricin, Shiga toxin/SLT-I, and SLT-II is targeted to a specific adenine residue of the 28S ribosomal RNA, thereby inactivating eucaryotic protein synthesis (Ito et al, 1987, 1988). In a recent study by Saxena et al. (1989), SLT-IIv was found to cleave the same adenine residue in Xenopus oocytes as Shiga toxin and ricin. Hovde et al. (1988) demonstrated that glutamic acid 167 in the A subunit of Shiga toxin/SLT-I is critical for the enzymatic activity of the SLT-I A subunit. This glutamic acid residue, which is conserved in SLT-IIv and SLT-II (Jackson et al., 1987a; Weinstein et al., 1988; this study), is within one of the regions of SLT-IIv which shared 75% homology with ricin. In a recent study, this same residue was demonstrated to be critical for the enzymatic activity of SLT-II (Jackson, M. and S. Calderwood, unpublished observations).

III. Role of the B subunit in cell specificity and extracellular localization.

Previous studies have revealed that the B subunits of Shiga toxin and the Shiga-like toxins are responsible for binding to a eucaryotic cell receptor (Gb_3 for Shiga toxin/SLT-I and SLT-II; Olsnes et al., 1981; Donohue-Rolfe et al., 1984). The third goal of this dissertation project was to determine whether the SLT-IIv B subunit is responsible for the differential cell specificity of SLT-IIv compared to the other members of the Shiga toxin family. A corollary to this goal was to examine the role of the various SLT B subunits in determining the levels of, as well as distribution, of cytotoxic activity (cell associated versus extracellular).

The results obtained from analysis of hybrid toxins produced by complementation and operon fusions support the hypothesis that the binding of SLT-IIv to a eucaryotic receptor(s) is qualitatively and/or quantitatively different than the other SLTs. SLT-IIv, unlike SLT-II, did not bind the Gal-Gal-BSA receptor analogue even though deduced amino acid sequence analyses (see preceding section and Weinstein et al., 1988) revealed that the the two toxins share considerable homology (94% for the A subunits and 84% for the B subunits). Recent data indicates that SLT-IIv does bind to Gb_3, but at a significantly reduced level when compared to SLT-II, and that SLT-IIv but not SLT-II binds to a larger glycolipid (J. E. Samuel, D. Weinstein, V. Ginsburg, A. D. O'Brien, and H. C. Krivan, manuscript in preparation). Taken together, these recent findings

suggest that SLT-IIv may have a different functional receptor(s) than does Shiga toxin/SLT-I or SLT-II.

The unique binding specificity of SLT-IIv may be due to specific amino acid sequence differences in the B subunit when compared to Shiga toxin/SLT-I and SLT-II (Weinstein et al., 1988; Gyles et al., 1988). The study described herein demonstrated that the B subunits of Shiga toxin and the Shiga-like toxins dictate the cytotoxic specificity. Regardless of the source of the A subunit, hybrid toxins comprised of the Shiga toxin/SLT-I B or SLT-II B subunit were equally cytotoxic for both Vero and HeLa cells. In contrast, hybrid cytotoxins comprised of any A subunit and the SLT-IIv B subunit had the same cytotoxicity profile as SLT-IIv holotoxin, i.e. they killed Vero cells with no detectable activity on HeLa cells.

Amino acids in the SLT-IIv B subunit were changed by site-directed mutagenesis and cloning to the residue(s) found in the corresponding position of Shiga toxin/SLT-I and SLT-II. One or two amino acids were changed at a time. None of the mutant SLT-IIv toxins displayed a different cytotoxicity specificity (i.e. killed HeLa cells as well as Vero cells). These results indicate that either more than two amino acid residues are critical for the different binding specificity of SLT-IIv from Shiga toxin/SLT-I and SLT-II or the residues targeted for change were not the correct ones. A recent study by Jackson (manuscript in preparation) supports the hypothesis that more than two amino acid residues are critical for the B subunit binding specificity. Jackson and his

coworkers used oligonucleotide-directed site-specific mutagenesis to change one or two specific amino acids in the B subunit of Shiga toxin. None of the mutations designed to make the Shiga toxin B subunit more homologous to the SLT-IIv B subunit resulted in a binding pattern similar to SLT-IIv (i.e. killed Vero cells significantly better than HeLa cells).

All of the hybrid cytotoxins produced by complementation or operon fusions were neutralized by polyclonal antisera specific for either Shiga toxin/SLT-I or SLT-II. This observation is in contrast to a recent study by Ito et al. who found that hybrid cytotoxins containing SLT-I and SLT-II components were not neutralized by polyclonal antisera. One explanation for this difference in neutralization results is that our antisera were raised against native holotoxin, whereas the antitoxins used by Ito et al. (1988) were raised against formalin-treated SLT-I or SLT-II. Perhaps the epitopes exposed in the hybrid toxins are better recognized by antisera to native toxin than antisera raised against the formalin-treated toxin.

In the same study by Ito et al. (1988), these investigators were able to detect a hybrid toxin composed of the SLT-I A and SLT-II B subunits, which produced levels of cytotoxic activity similar to a toxin composed of homologous subunits. I was also able to detect cytotoxic activity when the Shiga toxin/SLT-I A subunit gene was combined with a heterologous B subunit in vivo, either by subunit complementation or operon fusion. However, the activity of the hybrid SLT-IA/SLT-IIB toxins produced in my study was

reduced by orders of magnitude compared to the levels reported by Ito et al. One explanation for these discrepant findings on the cytotoxic activity of the hybrid toxins is that assembly of the heterologous A and B subunits of SLT-I and SLT-II using the in vivo methods described in this report may have resulted in predominantly a biologically inactive molecule for reasons that are unclear. That assembly does occur was demonstrated by L. Perera who used monoclonal antibodies to the A subunit and precipitated both the A and B subunits. What remains to be determined is whether the hybrid molecules that are assembled have the same A:B subunit ratio as the native toxin.

Previous studies have demonstrated that in lysogens of SLT-I or SLT-II-converting coliphages, SLT-I is predominantly cell-associated while SLT-II is found in equal amounts in the cell-associated and extracellular fractions (Strockbine et al., 1986). In E. coli producing SLT-IIv, most of the cytotoxin is localized to the extracellular milieu (this study; Weinstein et al., 1988). As shown in this study, cytotoxic specificity and localization of the hybrid cytotoxins corresponded to the source of the B subunit, regardless of the source of the A subunit. Hybrid cytotoxins containing the Shiga/SLT-I B subunit were almost exclusively cell-associated (>99%), and hybrid cytotoxins containing the SLT-II B subunit were predominantly cell-associated (86%). In contrast, hybrid cytotoxins containing the SLT-IIv B subunit were predominantly extracellular (94%). In this study, glutamine 64 in the B subunit of SLT-IIv was found to be critical for the

extracellular localization of the toxin.

SLT-IIv and SLT-II are associated with organisms that produce moderate levels of cytotoxin (Marques et al., 1986; Strockbine et al.,1986), while Shiga toxin/SLT-I is associated with organisms that produce high levels of cytotoxin. Explanations for the lower levels of cytotoxin produced by the SLT-II and SLT-IIv strains include: i) strains producing SLT-II and SLT-IIv may produce more inactive toxin than strains producing Shiga/SLT-I; ii) the slt-IIv and slt-II promoters may be weaker than the stx/slt-I promoters; or iii) on Vero cells, the affinity of the SLT-IIv and SLT-II B subunits for the cellular receptor may be lower than the affinity of the Shiga toxin/SLT-I B subunit. None of the above explanations can be ruled out by the data presented here. However, it is important to note that hybrid toxins that were regulated by the identical promoter produced different levels of cytotoxin depending on the source of the B subunit [i.e. compare the cytotoxic activity pFUS4 and pFUS2 (Table 12)].

The levels of native and hybrid cytotoxins produced by subunit complementation and operon fusions were not always equivalent, nor were the levels of analogous hybrid toxins produced by the two different methods. The total CD_{50} produced by transformants of the parental control plasmids were sometimes higher than transformants of the hybrid plasmids (eg. compare pMJ330 to pFUS2 in Table 12). The reasons for these variations in levels of cytotoxicity are not clear but may reflect the way in which active molecules are assembled. Also the total cytotoxicity of hybrid toxin produced

by subunit complementation was sometimes different than the levels produced by operon fusions which combined the same A and B subunits [i.e. compare the CD_{50} of the hybrid toxin comprised of SLT-IIv A/SLT-II B subunit produced by complementation (Table 11) to pFUS1 (SLT-IIv A/SLT-II B; Table 12)]. The production of hybrid toxins with an elevated CD_{50} may result from higher plasmid copy number (e.g., more copies of the subunit gene or hybrid operon) or the enhanced transcription of the gene or operon from an extragenic vector promoter.

IV. Creation of non-toxinogenic E. coli S1191.

The fourth goal of this dissertation project was to attempt to create a non-toxic, isogenic derivative of E. coli S1191. A role for SLT-IIv in edema disease has not been proven to date. It has been proposed that SLT-IIv produced in the gastro-intestinal tract of swine becomes disseminated (perhaps after damage to the gastric mucosa) and a toxinosis ensues. The neurological signs seen in ED may reflect damage to vascular endothelial cells in the central nervous system of swine. This hypothesis is based on the results of studies in which mice and rabbits were given Shiga toxin. The resulting neurological signs in these animals were secondary to the damage to vascular endothelial cells (Bridgwater et al., 1955; Howard, 1955).

A nontoxinogenic variant of an ED-causing strain of E. coli could be used in animal models to determine whether SLT-IIv is responsible for, or contributes to, the signs of edema disease. One approach to create such a strain is to replace the wild type

toxin gene with an inactivated one. One method for gene replacement involves cloning the mutated gene into a suicide vector which can not survive in the host strain, thereby forcing recombination. Another method for promoting gene replacement involves introducing a plasmid which carries the mutant gene into the wild type strain, subculturing the strain without selective pressure for the plasmid, and then screening for clones in which the wild type gene has been replaced by the mutant gene. The mutant gene can also be introduced by generalized and specialized transducing bacteriophage. Selection for mutants is simplified if the plasmid, phage, or transposon carries a selectable marker (eg. antibiotic resistance) and if there is an assay for the desired phenotype. The efficiency of recombination should increase with an increase in the amount of homologous DNA flanking the mutant gene. To encourage a recombinational event, the loss of the plasmid vector can be enhanced by co-introducing a second plasmid of the same incompatibility group into the strain. If the vector is resistant to a bacteriostatic antibiotic, such as chloramphenicol, clones which have lost the vector can be selected by ampicillin or D-cycloserine cycling (described in methods and results sections).

In this study, several subclones with mutant slt-IIv genes were constructed for the creation of a non-toxinogenic variant of an ED-causing E. coli. However, transformation (at a very limited frequency) was the only means I found to introduce foreign DNA into the wild type E. coli strain S1191. Therefore, I could not

introduce a transposable element to insertionally inactivate the slt-IIv chromosomal genes by transduction or conjugation. In addition, the relative resistance of strain S1191 to tetracycline, chloramphenicol, ampicillin and D-cycloserine limited the techniques and vectors which I could use for the construction of an SLT-IIv negative strain. If this non-toxinogenic strain is to be created, either more efficient transformation methods will need to be developed or a different type of vector system will need to be used.

SUMMARY

In conclusion, analysis of the mini-Mu lac operon fusions confirmed the location of the slt-I A and slt-I B genes, the direction of the transcription, and the existence of a second promoter for the slt-I B gene 3' to the slt-I A gene. Although, nucleotide sequence analyses revealed that the slt-IIv operon has a similar organization as the stx/slt-I and slt-II operons, the regulation of transcription of the operons differs. SLT-I production in E. coli, like Shiga toxin production in S. dysenteriae type 1, was found to be suppressed by iron, whereas SLT-IIv and SLT-II production in E. coli were not affected by iron levels. In addition, Shiga toxin production in S. dysenteriae 1 strain 60R was found to be regulated by temperature.

Data presented in this dissertation support the hypothesis that the different cytotoxic specificity of SLT-IIv as compared to Shiga toxin/SLT-I and SLT-II is due to differences in the B, or binding, subunit. A comparison of the deduced amino acid sequence of SLT-IIv with SLT-II revealed that the B subunits differ more than the A subunits. SLT-IIv did not bind a Gb_3-receptor analogue. In addition, hybrid toxins had the same cytotoxic specificities as the source of the B subunit, regardless of the source of the A subunit. The B subunit was also found to dictate the cellular localization after synthesis. While no single amino acid was identified as being essential for the binding to a eucaryotic receptor, glutamine 64 in the B subunit of SLT-IIv may be important for the extracellular localization of the toxin.

Finally, attempts were made to create a non-toxinogenic strain which was isogenic to the parent strain E. coli S1191. These strains were to be constructed to test the hypothesis that SLT-IIv is responsible for, or contributes to, the signs of ED. Although a nontoxinogenic strain was not isolated in this investigation, several plasmids were constructed that can be used in future studies to create such a strain.

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