

SHIGA-LIKE TOXIN TYPE II OF ENTEROHEMORRHAGIC  
ESCHERICHIA COLI (EHEC): GENETIC ORGANIZATION  
AND EFFECTS OF TOXIN IN A MURINE MODEL  
OF EHEC INFECTION

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SUNG



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MICROBIOLOGY

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## ABSTRACT

**Title of Dissertation:**

**Shiga-like Toxin Type II of Enterohemorrhagic Escherichia coli (EHEC): Genetic Organization and Effects of Toxin in a Murine Model of EHEC Infection**

**Lawrence M. Sung**

**Candidate, Doctor of Philosophy, 1990**

**Dissertation directed by:**

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**Professor, Department of Microbiology**

Enterohemorrhagic Escherichia coli (EHEC) strains associated with diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome in humans and E. coli strains responsible for edema disease of swine produce cytotoxins related to Shiga toxin of Shigella dysenteriae type 1. These Shiga-like toxins (SLTs) include Shiga-like toxin type I (SLT-I), Shiga-like toxin type II (SLT-II), and Shiga-like toxin type II variant (SLT-IIv) and constitute a family whose members are related in structure and biological activities.

The major objective of this study was to analyze the molecular genetics of Shiga-like toxin type II so as to better understand the relationship of SLT-II to other members of the Shiga toxin family.

One specific aim of this study was to characterize the SLT-II operon. The promoter was mapped by primer extension and S1 nuclease protection analyses, and the transcription terminator was identified by nucleotide sequence homology computer search. In contrast to the Shiga toxin and SLT-I operon promoters, the SLT-II operon promoter was not regulated by the fur gene product and its iron co-repressor. The transcriptional efficiency of the SLT-II operon promoter was equivalent to the Shiga toxin operon promoter under low iron growth conditions. Northern blot analysis revealed that the SLT-II operon was transcribed as a single polycistronic mRNA. These data indicated that the difference in the level of production between Shiga toxin/SLT-I and SLT-II as well as the single A subunit to multiple B subunit stoichiometry of the SLT-II holotoxin are not consequences of regulation at the transcriptional level.

Another specific aim of this research was to evaluate the virulence of E. coli strains containing recombinant Shiga-like toxin plasmids in an animal model. In contrast to Shiga toxin-producing bacteria, SLT-II-producing bacteria killed orally infected mice. Although the reason for the avirulence of Shiga toxin-producing bacteria was not determined, it was not due to the iron-regulation of Shiga toxin transcription or the efficiency of toxin release from the bacterial cell.

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GENETIC ORGANIZATION AND EFFECTS OF TOXIN IN  
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**by**

**Lawrence M. Sung**

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in partial fulfillment of the  
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Doctor of Philosophy 1990

## DEDICATION

I dedicate this dissertation to my father and mother. Since my earliest recollections, they have instilled me with the understanding that good is not good where better is expected. It is my fondest hope that I will be able to guide my children with the wisdom and caring of my parents and provide my family with the opportunities my father and mother have given me.

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# TABLE OF CONTENTS

<b>INTRODUCTION</b> . . . . .	1
<b>Preface</b> . . . . .	1
<b>I. Historical perspective</b> . . . . .	1
Shiga toxin of <u>Shigella dysenteriae</u> type 1 . . . . .	1
The Shiga-like toxins of <u>Escherichia coli</u> . . . . .	2
<b>II. Overview of the Shiga-like toxins</b> . . . . .	5
Biological activities . . . . .	5
Mode of action . . . . .	6
Functional characteristics . . . . .	7
<b>IIIa. Molecular biology of the Shiga-like toxins</b> . . . . .	10
Genetics . . . . .	10
Subunit structure and function . . . . .	11
Transcription of the SLT-I operon . . . . .	14
<b>IIIb. Comparison to other bacterial toxins</b> . . . . .	14
Cholera toxin and heat-labile toxin . . . . .	15
Diphtheria toxin . . . . .	16
<b>IVa. Association of Shiga-like toxins with disease</b> . . . . .	16
<b>IVb. Animal models</b> . . . . .	19
Streptomycin-treated mouse model . . . . .	21

Infection with laboratory strains . . . . .	22
<b>V. Specific aims of this dissertation . . . . .</b>	<b>22</b>
<b>MATERIALS AND METHODS . . . . .</b>	<b>24</b>
Bacterial strains and plasmids . . . . .	24
Media, enzymes, biochemicals, and radionuclides . . . . .	25
Preparation of plasmid DNA . . . . .	28
Transformation . . . . .	28
Oligonucleotide synthesis and nucleotide sequence analysis . . . . .	29
Cytotoxicity and iron effect assays . . . . .	29
Isolation of total cellular RNA . . . . .	29
Primer extension analysis . . . . .	32
S1 nuclease protection analysis . . . . .	33
Confirmation of the <u>slt-II</u> operon terminator . . . . .	34
Northern blot analysis . . . . .	35
Oligonucleotide-directed, site-specific mutagenesis . . . . .	36
Creation of promoter-deletion constructs . . . . .	37
Creation of SLT promoter / CAT transcriptional fusions . . . . .	37
Creation of SLT promoter / structural gene chimeras . . . . .	38
Mouse colonization experiments . . . . .	38

<b>RESULTS</b> .....	40
<b>I. Characterization of the SLT-II operon</b> .....	40
Promoter mapping of the SLT-II and SLT-IIv operons .....	40
Terminator mapping of the SLT-II operon .....	50
Transcriptional analyses of the SLT-II operon .....	53
Iron-regulation of SLT-II .....	53
SLT-II production in <u>E. coli</u> <u>crp</u> mutant backgrounds .....	60
<b>II. Functional analysis of the <u>slt-II</u>/<u>slt-IIv</u> promoter</b> .....	63
Functional confirmation of the <u>slt-II</u> and <u>slt-IIv</u> promoters .....	63
Comparison of transcriptional efficiencies of the <u>stx</u> and <u>slt-II</u> promoters .....	69
Shiga-like toxin promoter / structural gene chimeras .....	73
<b>III. Effects of Shiga-like toxin in a murine model of EHEC         infection</b> .....	77
Infection of mice with SLT-producing EHEC O157:H7 .....	77
Infection of mice with SLT-producing <u>E. coli</u> DH5 $\alpha$ .....	81
Effect of toxin release on the virulence of Shiga toxin- producing <u>E. coli</u> .....	84
Effect of iron on the virulence of Shiga toxin-producing <u>E. coli</u> .....	85

Infection of mice with <u>E. coli</u> DH5 $\alpha$ containing SLT subunit chimeras . . . . .	86
<b>DISCUSSION</b> . . . . .	89
I. <b>Transcriptional regulation studies</b> . . . . .	89
II. <b>Comparison of Shiga-like toxin promoter efficiencies</b> . . . . .	93
III. <b><u>In vivo</u> analysis of Shiga-like toxins</b> . . . . .	95
IV. <b>Summary</b> . . . . .	100
<b>LITERATURE CITED</b> . . . . .	101

## LIST OF TABLES

1.	Classifications of diarrheagenic <u>E. coli</u> . . . . .	3
2.	Summary of characteristics of members of the Shiga toxin family . . . . .	8
3.	Comparison of the processed Shiga-like toxin subunits . . . . .	12
4.	Recombinant plasmids used in this study . . . . .	26
5.	Oligonucleotides used in this study . . . . .	31
6.	Effects of iron on SLT-II production . . . . .	62
7.	Cytotoxicity of promoter deletion subclones in <u>E. coli</u> DH5 $\alpha$ . . . . .	68
8.	Shiga-like toxin promoter transcriptional efficiencies . . . . .	72
9.	Iron-regulation of Shiga-like toxin gene constructs . . . . .	76
10.	Effects of Shiga-like toxin-producing <u>E. coli</u> (Str <sup>f</sup> ) on streptomycin- treated mice . . . . .	79

## LIST OF FIGURES

1.	Primer extension analysis (PEII1) . . . . .	42
2.	Primer extension analysis (PEII3) . . . . .	44
3.	S1 nuclease protection analysis . . . . .	47
4.	Comparative nucleotide sequences of the Shiga-like toxins . . . . .	49
5.	Transcription terminator verification . . . . .	52
6.	Northern blot analysis (A/B probe) . . . . .	55
7.	Northern blot analysis (B only probe) . . . . .	57
8.	SLT lysogen / <u>fur</u> strain construction . . . . .	59
9.	Restriction endonuclease site creation . . . . .	65
10.	Shiga-like toxin promoter deletion subclone strategy . . . . .	67
11.	Shiga-like toxin promoter / <u>cat</u> transcriptional fusions . . . . .	71
12.	Shiga-like toxin promoter / structural gene chimera constructs . . . . .	75
13.	Competitive colonization assay (EHEC O157:H7 87-23 vs. <u>E. coli</u> DH5 $\alpha$ . . . . .	83
14.	Competitive colonization assay ( <u>E. coli</u> DH5 $\alpha$ vs. <u>E. coli</u> H1618) . . . . .	88

# INTRODUCTION

## **Preface**

This introduction consists of four sections. Part one contains an historical perspective of the Shiga-like toxins of Escherichia coli and an overview of our present understanding of the biological activities, mode of action, and functional characteristics of these toxins. The second section provides background information on the molecular genetics of the Shiga toxin family contemporary to this project. For comparison, a summary of findings on the genetics of other bacterial toxins is presented. The third section includes a discussion of the association between the Shiga-like toxins and human disease. This section also contains a description of animal systems currently available to examine infection by Shiga-like toxin-producing E. coli. Particular emphasis is placed on the murine model developed in this laboratory. The last part of the introduction details the specific aims of this dissertation.

## **I. Historical perspective.**

**Shiga toxin of Shigella dysenteriae type 1.** Shiga toxin has been recognized as one of the most potent bacterial toxins for eucaryotic cells. Shiga toxin was first described in 1903 by Conradi, who reported that intravenous inoculation of autolysates of Shigella dysenteriae type 1 paralyzed and killed

rabbits (Conradi, 1903). Subsequent reports demonstrated that crude preparations of Shiga toxin were cytotoxic for selected mammalian cells (Lasfarques and Delaunay, 1946, and Vicari *et al.*, 1960). Keusch *et al.* showed that partially purified Shiga toxin was enterotoxic, i.e., capable of eliciting fluid accumulation in ligated segments of rabbit ileum (Keusch *et al.*, 1972).

**The Shiga-like toxins of Escherichia coli.** Escherichia coli is one of several biological agents that can cause intestinal disease in humans and animals. Enterohemorrhagic E. coli (EHEC) strains constitute but one of five established categories for E. coli that cause diarrheal disease (as reviewed in Levine, 1987). The classifications are indicative of the pathogenic mechanisms of these bacteria (Table 1). Enterotoxigenic E. coli (ETEC) adhere to the mucosa of the small bowel and induce fluid secretion through the elaboration of two distinct enterotoxins designated heat-labile toxin (LT) and heat-stable toxin (ST) either singly or in combination. Enteroinvasive E. coli (EIEC) produce neither LT nor ST but, like Shigella, they penetrate and multiply within colonic epithelial cells. The role of enteroadherent E. coli (EAEC) in disease has not been proven, and strains of this classification are identifiable only by the patterns with which they adhere to Hep-2 cells. Enteropathogenic E. coli (EPEC) adhere avidly to the mucosa of the small intestine and produce a characteristic effacement of the microvilli. EPEC are not invasive like EIEC and do not produce LT or ST. EPEC have been further

Table 1. Classifications of diarrheagenic Escherichia coli <sup>a</sup>

Category	Acronym	Characteristic properties	Disease association
Enterotoxigenic	ETEC	adherent; and produce heat-labile toxin (LT) or heat-stable toxins (ST).	traveler's diarrhea
Enteroinvasive	EIEC	invasive (resembling <u>Shigella</u> ); and do <u>not</u> produce LT nor ST.	dysentery
Enteroadherent	EAEC	adherent to Hep-2 cells.	
Enteropathogenic	EPEC	adherent but not invasive like <u>Shigella</u> ; characteristic effacement of microvilli; and certain strains harbor EPEC adherent factor (EAF) plasmid.	infant diarrhea
Enterohemorrhagic	EHEC	<u>not</u> invasive like <u>Shigella</u> ; do <u>not</u> produce LT nor ST; produce Shiga-like toxins (SLTs); and produce intestinal lesions like EPEC.	hemorrhagic colitis hemolytic uremic syndrome

<sup>a</sup> adapted from Levine, 1987.

categorized based on the presence or absence of an EPEC adherence factor (EAF) plasmid.

EHEC strains are distinguished from other diarrheagenic E. coli in that they cause hemorrhagic colitis and the hemolytic uremic syndrome, possess a characteristic 60-megadalton plasmid that encodes the gene for fimbriae which can mediate attachment to Henle 407 intestinal cells, and belong to a restricted number of serotypes that include O157:H7, O26:H11, and O111:NM (as reviewed in Karmali et al., 1989). As with EPEC, EHEC are not classically invasive, do not produce LT or ST, but do produce characteristic effacement of the microvilli.

EHEC strains associated with diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome in humans and E. coli strains responsible for edema disease of swine produce elevated levels of cytotoxins functionally related to Shiga toxin of Shigella dysenteriae type 1. These cytotoxins have been termed Shiga-like toxins (SLTs) and include Shiga-like toxin type I (SLT-I), Shiga-like toxin type II (SLT-II), and Shiga-like toxin type II variant (SLT-IIv). The Shiga-like toxins are also referred to in the literature as Verotoxins (VTs).

The cytotoxic activity for Vero cells of culture filtrates of an E. coli O26:H11 strain, H30, was initially described by Konowalchuk et al. in 1977 (Konowalchuk et al., 1977). The enterotoxic activity of culture filtrates of another E. coli O26:H11 strain, H19, was reported earlier by Smith and Linggood (Smith and Linggood, 1971). Subsequently, O'Brien et al. demonstrated that the cytotoxic activity of

H30 culture filtrates could be neutralized by monospecific antibody to Shiga toxin (O'Brien *et al.*, 1982). O'Brien *et al.* established the "Shiga-like" toxin nomenclature and reported in 1983 that Shiga-like toxin and Verotoxin were synonymous (A.D. O'Brien, T.A. Lively, M.E. Chen, S.W. Rothman, and S.B. Formal, Letter, *Lancet i*: 702, 1983). In 1985, Scotland *et al.* discovered that *E. coli* O157:H7 strain 933 produced both Verotoxin type I (VT1), or SLT-I, and a second, immunologically distinct Verotoxin, or Shiga-like toxin, which Strockbine *et al.* later designated Shiga-like toxin type II (S.M. Scotland, H.R. Smith, and B. Rowe, Letter, *Lancet ii*: 885-886, 1985, and Strockbine *et al.*, 1986). A third Shiga-like cytotoxin, produced by edema disease-associated *E. coli* isolated from pigs, was identified in 1987 by Marques *et al.* and designated Shiga-like toxin type II variant (Marques *et al.*, 1987).

## II. Overview of the Shiga-like toxins.

**Biological activities.** The Shiga-like toxins possess biological activities similar to Shiga toxin (as reviewed in O'Brien and Holmes, 1987). The SLTs are paralytic and lethal for mice and rabbits, elicit fluid accumulation in ligated segments of rabbit ileum, and are cytotoxic for certain mammalian cells, which include HeLa, Vero, Daudi (human B lymphoma), KB, human liver, and human foreskin fibroblasts. SLT-IIv is classified as a variant of SLT-II because it is significantly more cytotoxic for Vero cells than for HeLa cells (Marques *et al.*,

1987).

**Mode of action.** The biological properties of Shiga toxin and the SLTs result from the inhibition of protein synthesis in susceptible target cells (Thompson *et al.*, 1976, Brown *et al.*, 1980, Reisbig *et al.*, 1981, and Obrig *et al.*, 1987). Like several other bacterial toxins such as diphtheria, pertussis, cholera, *E. coli* heat-labile toxin, and *Pseudomonas* exotoxin A (as reviewed in Middlebrook and Dorland, 1984), Shiga toxin is a subunit toxin comprised of an A (active) subunit and several B (binding) subunits (Olsnes *et al.*, 1981, and Donohue-Rolfe *et al.*, 1984). The general mode of action of such toxins involves binding to a specific receptor on the cell surface via the B subunits and then internalization of the A subunit which interrupts cell function through interaction with specific components of the subcellular machinery.

The eucaryotic receptor to which the B subunits of Shiga toxin, SLT-I, and SLT-II bind is globotriosyl ceramide (Gb<sub>3</sub>), a glycolipid containing a terminal disaccharide galactose- $\alpha$ (1 $\rightarrow$ 4)-galactose (Jacewicz *et al.*, 1986, Lindberg *et al.*, 1986, Lindberg *et al.*, 1987, Lingwood *et al.*, 1987, and Waddell *et al.*, 1988). In contrast, the B subunit of SLT-IIv preferentially binds globotetraosyl ceramide (Gb<sub>4</sub>) and globopentaosyl ceramide (Gb<sub>5</sub>), which suggests that the difference in the cell specificity of SLT-IIv noted above reflects a difference in receptor recognition (De Grandis *et al.*, 1989, and Samuel *et al.*, 1990).

Following receptor binding, the toxin is internalized by a specific receptor-mediated endocytotic event (as reviewed in O'Brien and Holmes, 1987). The proteolytic nicking and reduction of the A subunit within the endocytic vesicle creates an enzymatically active A<sub>1</sub> fragment, which is delivered to the cytosol by an unknown mechanism where it binds the 60S ribosomal subunit. The A<sub>1</sub> fragment possesses ribonucleic acid N-glycosidase activity which has been shown to cleave the N-glycoside bond in the adenosine residue at position 4324, in the case of rat liver ribosomes (Endo *et al.*, 1987, and Endo *et al.*, 1988), or at position 3732, in the case of *Xenopus* oocyte ribosomes (Saxena *et al.*, 1989), in 28S ribonucleic acid of the 60S ribosomal subunit. This enzymatic activity, identical to that of the plant toxin ricin, results in the inhibition of elongation factor 1 dependent aminoacyl transfer ribonucleic acid binding to the eucaryotic ribosome, the cessation of protein synthesis, and, ultimately, cell death.

**Functional characteristics.** Although the members of the Shiga toxin family exhibit similar biological activities, the Shiga-like toxins can be distinguished from one another by reactivity with monospecific and/or monoclonal antibodies, level and cellular location of cytotoxic activity, and response to iron concentrations in the growth environment (Table 2).

The Shiga-like toxins can be categorized into two antigenically distinct types. The first group includes Shiga toxin and SLT-I, which are neutralized by

Table 2. Summary of characteristics of members of the Shiga toxin family <sup>a</sup>

Toxin	Cytotoxicity CD <sub>50</sub> /ml	αST	Neutralized by αSLT-IB	αSLT-II	Location of structural genes	Disease association
Shiga	10 <sup>5</sup> - 10 <sup>8</sup>	+	+	-	Chromosome	Bacillary dysentery
SLT-I	10 <sup>5</sup> - 10 <sup>8</sup>	+	+	-	Phage	Diarrhea, HC, HUS
SLT-II	10 <sup>3</sup> - 10 <sup>4</sup>	-	-	+	Phage	Diarrhea, HC, HUS
SLT-III	10 <sup>3</sup> - 10 <sup>4</sup>	-	-	+	Chromosome	Edema disease of swine

<sup>a</sup> adapted from O'Brien and Holmes, 1987.

Abbreviations used: ST, Shiga toxin; HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; and CD<sub>50</sub>/ml, cytotoxic dose 50 % per milliliter.

anti-Shiga toxin and monoclonal antibodies to the SLT-I B subunit (O'Brien et al., 1982, O'Brien et al., 1983, and Strockbine et al., 1985). The second antigenic class of SLTs includes SLT-II and SLT-IIv, both of which are neutralized by polyclonal antisera against SLT-II but not by anti-Shiga toxin (Marques et al., 1987, and Perera et al., 1988).

The Shiga-like toxins also differ in both level and cellular localization of cytotoxic activity (as reviewed in O'Brien and Holmes, 1987). Shiga toxin and SLT-I are associated with organisms that produce high levels of cytotoxin, while SLT-II and SLT-IIv are associated with organisms that produce moderate levels of cytotoxin. SLT-II is about 1000-fold less cytotoxic per milligram protein cell lysate or per milliliter culture filtrate than is SLT-I. However, SLT-II is about 10-fold more potent as a mouse lethal toxin. In addition, SLT-II is distributed equally between the cell-associated and extracellular fractions, whereas SLT-I is almost entirely cell-associated. Shiga toxin is a periplasmic protein in *S. dysenteriae* type 1 and is thought to be released into the media upon autolysis.

Shiga toxin and SLT-I production have been shown by several groups to be repressed by high levels of iron (Dubos and Geiger, 1946, van Heyningen and Gladstone, 1953, McIver et al., 1975, O'Brien and LaVeck, 1982, and Weinstein et al., 1988a), while SLT-II and SLT-IIv production are not influenced by the concentration of iron in the growth media (Weinstein et al., 1988b). Iron-regulation of SLT-I production is mediated at the transcriptional level by the action of a

repressor complex on the SLT-I operon (Calderwood and Mekalanos, 1987).

### **IIIa. Molecular biology of the Shiga-like toxins.**

**Genetics.** The production of several important bacterial toxins, such as diphtheria toxin, botulinum toxin, streptococcal erythrogenic toxin, and staphylococcal enterotoxin A, is mediated by toxin-converting bacteriophages that contain the genes encoding the respective toxin (Barksdale and Arden, 1974, and Betley *et al.*, 1986). Sensitive bacterial hosts that are lysogenized by such toxin-converting phages acquire the capacity to produce the corresponding toxin. The production of SLT-I and SLT-II has been shown to be determined by specific phages in certain strains of EHEC serotypes isolated from humans. Bacteriophages from EHEC O26:H11 strains that code for SLT-I are morphologically distinct and have different host ranges and immunity specificities than SLT-II converting phages from EHEC O157:H7 strains (O'Brien *et al.*, 1984). Sequences on the genome of the SLT-I converting phage H19B from EHEC O26:H11 strain H19 are homologous to the region on the genome of phage lambda that encodes the origin of replication and the genes *cl*, *ninR*, *Q*, and *P* (Huang *et al.*, 1986). In contrast to that of SLT-I and SLT-II, the production of SLT-IIv is not controlled by lysogenic phages (Smith *et al.*, 1983).

The genes encoding the Shiga and Shiga-like toxins have been cloned in our laboratory (Newland *et al.*, 1985, Newland *et al.*, 1987, Strockbine *et al.*, 1988,

and Weinstein *et al.*, 1988b). Nucleotide sequence analyses indicate that sequences encoding the A subunit and B subunit polypeptides of the SLTs are arranged contiguously in an operon (Calderwood *et al.*, 1987, De Grandis *et al.*, 1987, Jackson *et al.*, 1987a, Jackson *et al.*, 1987b, Gyles *et al.*, 1988, Strockbine *et al.*, 1988, and Weinstein *et al.*, 1988b). There is an intercistronic gap varying between twelve and fifteen nucleotides in length, depending on the particular toxin operon. The structural genes for Shiga toxin and SLT-I, designated *stx* and *slt-I*, differ by only three nucleotides, which translates to a single conservative amino acid difference between the A subunits of the two toxins. These nearly identical toxins are thus referred to synonymously as Shiga toxin/SLT-I. The structural genes of SLT-II and SLT-IIv, designated *slt-II* and *slt-IIv*, share 55% and 60% nucleotide sequence homology, respectively, with *stx/slt-I*. A putative ribosomal binding site exists immediately upstream of the A subunit coding region of each of the Shiga-like toxins, with a second putative Shine-Dalgarno site located in the intercistronic gap.

**Subunit structure and function.** The sizes of the mature A subunit and B subunit polypeptides of the Shiga-like toxins as calculated from the deduced amino acid sequences are listed in Table 3. Analysis of the translated amino acid sequences indicates that the A and B polypeptides of the SLTs contain hydrophobic signal sequences at their amino termini and are probably synthesized as

Table 3. Comparison of the processed Shiga-like toxin subunits <sup>a</sup>

	Subunit	Nucleotides	Amino acid residues	Molecular Weight	pI <sup>b</sup>
A	Shiga toxin/SLT-I	879	293	32,225/32,211	11.1
	SLT-II	888	296	33,135	9.8
	SLT-IV	891	297	33,050	8.7
B	Shiga toxin/SLT-I	207	69	7,690	5.9
	SLT-II	210	70	7,817	5.4
	SLT-IV	204	68	7,565	10.2

<sup>a</sup> adapted from Strockbine *et al.*, 1988, and Weinstein *et al.*, 1988b.

<sup>b</sup> pI, isoelectric point.

precursor forms of secreted periplasmic proteins. Cross-linking experiments performed by Donohue-Rolfe et al. demonstrated that Shiga toxin is comprised of one A subunit and five B subunits (Donohue-Rolfe et al., 1984). The Shiga-like toxins also exhibit a single A to multiple B subunit association, although the exact protein stoichiometry remains unclear.

Individual subunits of SLT-I and SLT-II can assemble to form fully cytotoxic hybrid molecules in vitro, as demonstrated by Ito et al., with purified A and B subunits of the toxins (Ito et al., 1988). Experiments using subunit complementation and operon fusions to create hybrid cytotoxins in vivo, indicate that the cytotoxic specificity and localization of the toxin within the bacterial cell is dictated by the corresponding Shiga-like toxin B subunit (Weinstein et al., 1989).

Amino acid sequence comparison of the A subunits of Shiga toxin/SLT-I, SLT-II, and ricin reveal two regions of significant homology that lie within a proposed active-site cleft, identified by x-ray crystallography, on the ricin A polypeptide chain (Montfort et al., 1987). Site-directed mutagenesis studies have demonstrated that amino acid substitution of glutamic acid residue 167 of the SLT-I A subunit (Hovde et al., 1988) or glutamic acid residue 167 of the SLT-II A subunit (Jackson et al., in press) drastically reduces the enzymatic activity of these respective molecules.

**Transcription of the SLT-I operon.** A promoter located upstream of the slt-IA gene and a transcription terminator have been identified for the SLT-I operon (De Grandis et al., 1987). A 21 base pair region of dyad symmetry situated within the slt-I promoter sequences functions as a binding site for the Fur protein / iron co-repressor complex (Calderwood and Mekalanos, 1987). The decreased production of SLT-I in high iron growth conditions results from the interaction of this repressor complex with the slt-I promoter which inhibits transcription of the toxin genes.

Studies by De Grandis et al. on the SLT-I operon suggest that slt-IA and slt-IB are transcribed as a polycistronic mRNA. However, their study, and previous reports, did not preclude the possible existence of another functional promoter within the downstream sequences of slt-IA which could direct the independent transcription of the slt-IB gene (De Grandis et al., 1987, Newland et al., 1987, and Weinstein et al., 1988a). Northern blot analysis on the Shiga toxin operon by Kozlov et al. demonstrated both an A subunit / B subunit polycistronic mRNA and an independent B subunit transcript (Kozlov et al., 1988).

### **IIIb. Comparison to other bacterial toxins.**

The Shiga-like toxins share similar features at the genetic level with other toxins of bacterial origin. Analogies with respect to the location and arrangement of the SLT genes exist with a number of bacterial toxins. In particular, there are

three toxins which can serve as paradigms for our understanding of the expression and regulation of the genes for the Shiga-like toxins.

**Cholera toxin and heat-labile toxin.** The structure and genetic organization of both cholera toxin (CT) and *E. coli* heat-labile toxin (LT) closely resemble those of the Shiga-like toxins. The CT and LT molecules are also bipartite toxins, composed of five B subunits associated with one A subunit (as reviewed in Betley *et al.*, 1986). Genes coding for the respective subunits of CT and LT are arranged contiguously in an operon, although, in contrast to the SLT genes, the A subunit and B subunit cistrons overlap. Transcription occurs as a polycistronic message initiated at a single promoter upstream of the A coding region. The first of two translational initiation signals which exist for the CT and LT operons is located upstream of the A gene. A second Shine-Dalgarno site preceding the B subunit gene is located within the coding region for the A subunit of both the CT and LT operons. A fusion that placed *ctxB* under the control of the translational signals of *ctxA* was found to synthesize nine times less B subunit than the wild-type gene, which suggests that the ribosomal binding site for the B subunit promotes translation more efficiently than the ribosomal binding site for the A subunit in CT and LT (Mekalanos *et al.*, 1983). There is also evidence to suggest that translation of the A subunits of CT and LT occurs less efficiently than that for the B subunits of these toxins because of a predominance in the A subunit

message of stable local secondary structures which interfere with ribosome movement (Yamamoto et al., 1985). Therefore, in CT and LT, regulation at the level of translation, not transcription, permits the synthesis of A subunits and B subunits in relative amounts appropriate to account for the subunit stoichiometry observed in the respective holotoxins.

**Diphtheria toxin.** Like SLT-I, diphtheria toxin of Corynebacterium diphtheriae is iron-regulated and has served as a model for the regulation of a phage-encoded toxin gene by iron (Murphy et al., 1976). Expression of the diphtheria toxin operon of the  $\beta$  phage of C. diphtheriae is, like SLT-I, regulated at the level of transcription. This regulation occurs through the action of a corynebacterial regulatory protein, analogous to the Fur protein of E. coli, and its iron co-repressor (Tai and Holmes, 1988, and Fourel et al., 1989). In the future, information derived from studies on the iron-regulation of diphtheria toxin may provide insight as to the significance of such a regulatory mechanism within the Shiga toxin family.

#### **IVa. Association of Shiga-like toxins with disease.**

There is no direct evidence that Shiga toxin and Shiga-like toxins alone act as virulence factors in either natural or experimental infections. However, the epidemiological association of the SLTs with E. coli strains isolated from humans

and animals clearly implicates a role for these toxins in disease. SLT-producing EHEC O157:H7 strains have consistently been linked with both sporadic cases and outbreaks of several well-defined syndromes: hemorrhagic colitis (HC), the hemolytic uremic syndrome (HUS), and, more recently, thrombotic thrombocytopenic purpura (TTP) (Riley *et al.*, 1983, Wells *et al.*, 1983, Karmali *et al.*, 1985, and Morrison *et al.*, 1985). In addition, *E. coli* strains associated with edema disease and post-weanling diarrhea in pigs usually produce SLT-IIv (Marques *et al.*, 1987).

Hemorrhagic colitis is a distinct clinical syndrome that typically presents with abdominal cramps and watery diarrhea followed by a hemorrhagic discharge resembling lower gastrointestinal bleeding (as reviewed in Riley, 1987). The hemolytic uremic syndrome is a clinical entity defined by a triad of features which include acute renal failure, thrombocytopenia, and a microangiopathic hemolytic anemia (Gasser *et al.*, 1955). Classical HUS has its highest incidence in infants and young children and is usually preceded by bloody diarrhea. This acute prodromal illness shows remarkable clinicopathologic similarities with hemorrhagic colitis which suggests that both syndromes are manifestations of the same underlying disease process. Thrombotic thrombocytopenic purpura differs from HUS only in that neurological signs and fever are more prominent in TTP and the peak age of incidence is in the third decade of life rather than the first decade (Moschowitz, 1924).

The histopathology of these syndromes is characterized by widespread sterile systemic microangiopathic lesions consistent with a systemic toxemia. Because there have been no reports in the literature that demonstrate SLTs in the blood of patients with hemorrhagic colitis or HUS, there is no direct proof that Shiga-like cytotoxins are responsible for this vascular pathology. However, there are data to indicate that Shiga-like toxins are produced in vivo and do stimulate an immune response. Karmali et al. found that patients with EHEC infections that progressed to HUS developed a four-fold or greater rise in neutralizing antibodies to SLT-I (Karmali et al., 1985). Keusch et al. showed that anti-Shiga toxin neutralizing antibodies developed in human sera both after natural infections with S. dysenteriae type 1 and after experimental infections of volunteers (Keusch et al., 1976). Aside from EHEC, S. dysenteriae type 1 is the only other microbe for which a convincing association exists with HUS. Karmali et al. have suggested that Shiga toxin/SLT-I may be the common denominator responsible for the association of these organisms with HUS (Karmali et al., 1985).

No report has clearly demonstrated the production of antibodies to SLT-II after natural or experimental infections, presumably because SLT-II is produced in much lower quantities than Shiga toxin or SLT-I. The fact that SLT-II is produced at lower levels than SLT-I in vitro and perhaps in vivo is puzzling in light of recent reports that EHEC O157:H7 strains containing genes for SLT-II alone are more likely to cause a diarrheal syndrome progressing to HUS or TTP than strains

which contained genes for both SLT-II and SLT-I or SLT-I alone (Scotland *et al.*, 1987, and Ostroff *et al.*, 1989). That EHEC O157:H7 isolates with only the SLT-II genes are more likely to produce HUS and TTP than strains which carry both SLT-I and SLT-II is particularly perplexing.

It has been proposed that the glomerular damage in humans is a consequence of the cytotoxic effect of Shiga-like toxin on capillary endothelial cells. Studies using human umbilical vein endothelial cells have demonstrated that purified Shiga toxin has a direct cytotoxic effect on vascular endothelial cells in cultures (Obrig *et al.*, 1988). Injection of Shiga toxin or SLT into animals caused ischemic changes and endothelial cell damage with microangiopathy, similar to that seen in human patients with HUS (Richardson *et al.*, 1987, and Richardson, S.E., V. Jagadha, C.R. Smith, L.E. Becker, M. Petric, and M.A. Karmali, Abstr. Int. Symp. Workshop Verocytotoxin-Producing *Escherichia coli* Infections 1987, AMV-5). Observations of the direct effects of the Shiga-like toxins in animals constitute much of the suggestive evidence for the role of SLTs in human disease.

#### **IVb. Animal models.**

Several animal models have been developed for studying the virulence mechanisms of EHEC O157:H7 and other *E. coli* strains that produce elevated levels of Shiga-like toxins. The colonization and induction of disease following oral infection with Shiga-like toxin-producing EHEC O157:H7 strains have been

studied in the infant rabbit, the gnotobiotic piglet, and, most recently, the streptomycin-treated mouse.

When infant rabbits were inoculated intragastrically with  $10^8$  EHEC O157:H7 cells, animals consistently developed diarrhea (Pai *et al.*, 1986). The damage to the mucosal epithelium was seen mainly in the mid- and distal colon and was characterized by increased mitotic activity in the crypts, mucin depletion, a mild to moderate infiltration of neutrophils in the lamina propria and epithelium, and apoptosis in the surface cell epithelium. Apoptosis (individual cell death) is a striking feature of the histopathological damage to adult rabbit ileum after exposure to purified Shiga toxin or SLT-I (Keenan *et al.*, 1986). Pai *et al.* further demonstrated using infant rabbits that the mucosal abnormalities of rabbits inoculated with partially purified toxin alone mimic those seen when the intact organisms are used.

Oral infection of the gnotobiotic piglet by EHEC O157:H7 produced attach-and-effacement lesions characteristic of EPEC and EHEC in the ceca and colons of the animals (Tzipori *et al.*, 1986). The severity of diarrhea correlated with the extent and distribution of attachment and effacement. The most virulent strains examined in Tzipori *et al.*'s study were those that caused attaching-effacing lesions in the proximal small intestine as well as the rest of the gastrointestinal tract. The attaching-effacing lesions of the isolates that caused little or no diarrhea were primarily restricted to the large intestine and the distal small

intestine. Although most EHEC strains contain a characteristic 60-megadalton plasmid, no role for the plasmid could be demonstrated in the colonization or disease of gnotobiotic piglets (Tzipori et al., 1987).

A recent report described the use of streptomycin-treated mice to examine the colonization of an animal host with EHEC O157:H7 (Wadolkowski et al., manuscript submitted). The streptomycin-treated mouse model was used in this dissertation project to assess the in vivo effects of Shiga-like toxins elaborated from laboratory E. coli K-12 strains.

**Streptomycin-treated mouse model.** A mouse-passaged SLT-producing EHEC O157:H7 strain cured of the 60-megadalton plasmid killed orally infected streptomycin-treated mice within five days of feeding (Wadolkowski et al., manuscript submitted). In marked contrast to the edematous necrotic lesions noted in rabbit models and in the gnotobiotic piglet, no gross or histologic lesions were observed in the colons of these animals. Thus, the death of these mice was not due to hemorrhagic colitis. Rather, histopathological examination of various body tissues revealed that death of the orally infected mice was due to acute renal cortical tubular necrosis. One of the objectives of this dissertation project was to assess the role of the Shiga-like toxins in the tubular damage leading to the death of these mice.

**Infection with laboratory strains.** E. coli K-12 laboratory strains colonize the bowel of normal animals or humans poorly (about  $10^6$  cells per gram feces) relative to normal E. coli fecal isolates (about  $10^8$  cells per gram feces) (Laux et al., 1982). Furthermore, such K-12 strains usually persist for only a few days. However, if selective pressure, such as treatment with antibiotics, is applied to favor antibiotic-resistant E. coli K-12 strains these bacteria will colonize humans and animals well and persist indefinitely. These findings indicate that laboratory E. coli K-12 strains can colonize the large intestine of humans and animals, but they colonize poorly under normal conditions because the indigenous flora of the host has a selective advantage over strains introduced exogenously.

## V. Specific aims of this dissertation

The major objective of the research constituting this dissertation was to analyze the molecular genetics of Shiga-like toxin type II of enterohemorrhagic Escherichia coli so as to better understand the relationship of SLT-II to other members of the Shiga toxin family. Therefore, the first specific aim of this project was to characterize the SLT-II operon. This characterization involved mapping the promoter and transcription terminator, determining whether specific regulatory proteins such as the cyclic adenosine monophosphate (cAMP) regulatory protein (CRP) or the ferric uptake regulator (Fur) were involved in slt-II transcription, and analyzing the transcription products. As a result of information obtained in the

first specific aim, subsequent efforts centered on the genetic manipulations of the promoters for the Shiga-like toxin operons.

The second specific aim was to compare the transcriptional efficiencies of the slt-II and stx promoters. This involved the creation of restriction endonuclease sites in the Shiga toxin and SLT-II operons to enable the isolation and study of the respective toxin promoters. These newly created restriction sites permitted the functional confirmation of the slt-II promoter by deletion subcloning, the comparison of slt-II and stx promoter activity in a promoter analysis vector, and the construction of operon chimeras in which toxin structural genes were fused to heterologous SLT promoters.

The third specific aim of this dissertation project was to evaluate the virulence of E. coli strains containing recombinant Shiga-like toxin plasmids in the orally infected streptomycin-treated mouse. These experiments were designed to test the role of Shiga-like toxins in pathogenesis and to assess the significance of iron-regulation of toxin in vivo using the promoter / structural gene chimeras created in the second specific aim.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* HB101 ( $F^-$  hsdS20( $r_B^-$   $m_B^-$ ) recA13 ara-14 proA2 lacY1 galK2 rpsL20( $Sm^r$ ) xyl-5 mtl-1 supE44  $\lambda^-$ ) and *E. coli* DH5 $\alpha$  ( $F'$   $\phi$ 80dlacZ  $\Delta$ (lacZYA-argF) U169 recA1 endA1 hsdR17( $r_K^-$   $m_K^+$ ) supE44  $\lambda^-$  thi-1 gyrA relA1) were used as hosts for transformation of recombinant plasmids (Boyer and Roulland-Dussoix, 1969, and Bethesda Research Laboratories *Focus*, 1986, 8:2, 9). *E. coli* JM109 (endA1 gyrA96 thi hsdR17 supE44 relA1 traD36  $\Delta$ (lac proAB)/ $F'$  proAB lacI<sup>q</sup>Z M15) and *E. coli* CJ236 (dut ung thi-1 relA1 / pCJ105 ( $Cm^r$   $F'$ )) were used as hosts for the propagation of bacteriophage M13 (Yanisch-Perron *et al.*, 1985, and Bio-Rad Laboratories, Richmond, CA). *E. coli* strains AB2847, H1618, and H1646 were kindly provided by Dr. Klaus Hantke, Mikrobiologie II, Universität Tübingen, Tübingen, FRG; *E. coli* strain JE5505 was kindly provided by Dr. Henry C. Wu, Department of Microbiology, Uniformed Services University of the Health Sciences; and *E. coli* strains JK7 and JK7(pHA5) were kindly provided by Dr. Anthony T. Maurelli, Department of Microbiology, Uniformed Services University of the Health Sciences. These strains have all been described elsewhere (Hantke, 1984; Hirota *et al.*, 1977; and Aiba *et al.*, 1982). The SLT-I converting phage H19J and the SLT-II converting phage 933W have also been described previously (Smith *et al.*, 1983, O'Brien *et al.*, 1984, and Strockbine *et al.*, 1986). The recombinant plasmids used in this study

are listed in Table 4. Studies using strains transformed with pNAS13 were performed under BL3+EK1 containment conditions prescribed by the guidelines of the National Institutes of Health Recombinant DNA Advisory Committee (Federal Register, 1986). The following plasmids were used as vectors for cloning as well as templates for sequencing and oligonucleotide-directed, site-specific mutagenesis: pBR329 (Boehringer Mannheim Biochemicals, Indianapolis, IN), pBS and pBluescript KS (Stratagene, La Jolla, CA), and the M13 mp18 and mp19 replicative form vectors (New England BioLabs, Inc., Beverly, MA). The following additional plasmids were used for promoter and transcription terminator analyses: pKK232-8 and pCM4 (Pharmacia LKB Biotechnology, Piscataway, NJ).

**Media, enzymes, biochemicals, and radionuclides.** Luria broth or Luria agar (Maniatis *et al.*, 1982) was used for routine culturing of bacteria. For iron-regulation studies, Chelex-(Bio-Rad Laboratories) treated 2% glucose syncase was used with or without the addition of 10  $\mu\text{g}$  of  $\text{Fe}^{3+}$  per ml in the form of  $\text{FeCl}_3$ . Where required, media were supplemented with the following antibiotics: ampicillin (100  $\mu\text{g}/\text{ml}$ ), streptomycin (50  $\mu\text{g}/\text{ml}$ ), chloramphenicol (50  $\mu\text{g}/\text{ml}$ ), and tetracycline (12.5  $\mu\text{g}/\text{ml}$ ).

Restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA ligase, calf intestinal phosphatase, RNase A, Maloney-murine leukemia virus reverse transcriptase, and S1 nuclease were purchased from Boehringer Mannheim

Table 4. Recombinant plasmids used in this study

Plasmid	Toxin	Description	Reference or source
pNAS13	Shiga	pBR329 with entire <u>stx</u> operon	(Strockbine <u>et al.</u> , 1988)
pNN103	SLT-II	pBR328 with entire <u>slt-II</u> operon	(Newland <u>et al.</u> , 1987)
pMJ100	SLT-II	pBS with entire <u>slt-II</u> operon	(Weinstein <u>et al.</u> , 1989)
pMJ330	SLT-II	pMJ100 with created <u>HpaI</u> site between <u>slt-IIA</u> and <u>slt-IIB</u>	<i>ibid.</i>
pDLW5	SLT-IV	pBR329 with entire <u>slt-IV</u> operon	<i>ibid.</i>
pDLW5.125	SLT-IV	pDLW5 with created <u>EcoRI</u> site downstream of promoter	(Weinstein, D.L.)
pLMS0.0	Shiga	pNAS13 with created <u>BamHI</u> site downstream of promoter	this study
pLMS1.0	SLT-II	pNN103 with created <u>HpaI</u> and <u>BamHI</u> sites bracketing promoter	this study
pLMS2.1	SLT-II	pLMS1.0 subclone (into pBS) deleting <u>slt-II</u> promoter	this study
pLMS2.2	SLT-II	pLMS1.0 subclone (into pBS) retaining <u>slt-II</u> promoter	this study
pLMS2.3	SLT-IV	pDLW5.125 subclone (into pBS) deleting <u>slt-IV</u> promoter	this study

Table 4 (continued). Recombinant plasmids used in this study

Plasmid	Toxin	Description	Reference or source
pLMS4.0	none	pBS with <u>cat</u> gene cassette of pCM4	this study
pLMS4.4	none	pLMS4.0 with <u>slt-II</u> terminator between <u>lac</u> promoter and <u>cat</u>	this study
pLMSCATI	none	pKK232-8 with <u>stx</u> promoter / <u>cat</u> transcriptional fusion	this study
pLMSCATII	none	pKK232-8 with <u>slt-II</u> promoter / <u>cat</u> transcriptional fusion	this study
pLMSCATC	none	pKK232-8 with <u>lac</u> promoter / <u>cat</u> transcriptional fusion	this study
pFUS4	hybrid	pBR329 with <u>slt-IIA/stxB</u> operon fusion	(Weinstein et al., 1989)
pFUS6	hybrid	pBR329 with <u>slt-IIvA/stxB</u> operon fusion	ibid.
pLMS40	hybrid	pBS with insert as pFUS4	this study
pLMS60	hybrid	pBS with insert as pFUS6	this study
pLMS100	SLT-II	pBR329 with <u>stx</u> promoter / <u>slt-II</u> structural gene fusion	this study
pLMS200	Shiga	pBR329 with <u>slt-II</u> promoter / <u>stx</u> structural gene fusion	this study

Biochemicals. The RNase inhibitor, RNasin, was purchased from Promega Corp., Madison, WI. Lysozyme was purchased from Sigma Chemical Co., St. Louis, MO. Agarose for DNA and RNA electrophoresis was purchased from International Biotechnologies, Inc., New Haven, CN. Nick translation kits were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Sequenase DNA sequencing kits were purchased from U.S. Biochemicals Corp., Cleveland, OH. The non-radioactive chloramphenicol acetyltransferase (CAT) enzyme assay kit was purchased from 5 Prime to 3 Prime, Inc., West Chester, PA. Muta-Gene DNA mutagenesis kits were purchased from Bio-Rad Laboratories. Radionuclides were purchased either from Dupont, NEN Research Products, Boston, MA or from Amersham Corp., Arlington Heights, IL.

**Preparation of plasmid DNA.** Rapid isolations and large-scale preparations of plasmid DNA were done by methods outlined by Maniatis *et al.*, 1982. When appropriate, individual restriction fragments were isolated by electroelution by following the instructions supplied by the manufacturer of the electroelution apparatus (International Biotechnologies Inc.).

**Transformation.** Bacteria were transformed with ligation mixtures or purified plasmid DNA using the  $\text{CaCl}_2$  methods described by Hanahan *et al.*, 1983, or by electroporation methods described by the manufacturer (Bio-Rad

Laboratories).

**Oligonucleotide synthesis and nucleotide sequence analysis.** Synthetic oligonucleotides listed in Table 5 were prepared with a model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Nucleotide sequence analysis was done by the dideoxy chain termination method (Sanger *et al.*, 1977) following the procedure provided by the supplier of the Sequenase DNA sequencing kit (U.S. Biochemicals Corp.).

**Cytotoxicity and iron effect assays.** Microcytotoxicity assays were done on Vero cells according to published modifications (Marques *et al.*, 1986) of the methods of Gentry and Dalrymple, 1980. The last dilution of the sample in which greater than or equal to 50% of the Vero cells detached from the plastic as assessed by  $A_{620}$  measurements was considered the 50% cytotoxic dose ( $CD_{50}$ ). Tests for regulation of cytotoxin production by iron were performed as described by Weinstein *et al.*, 1988.

**Isolation of total cellular RNA.** Total cellular RNA was isolated by a modification of the guanidinium isothiocyanate RNA extraction procedure (Chirgwin *et al.*, 1979). Bacterial cultures (40 ml) were grown to mid-logarithmic phase and harvested by centrifugation, and the bacterial pellet was resuspended in 10 ml of

### Table 5 Legend

- a The oligonucleotides (with the exception of SLTII9) are reverse complementary to the published nucleotide sequence of the strand coding for the respective toxin structural genes, and therefore represent the noncoding strand sequence. SLTII9 represents the coding strand sequence.
- b The positions of the oligonucleotides (with the exception of SLTII9) are listed relative to the initiation codon (+1 adenine) for the respective toxin structural gene. SLTII9 is located 124 to 139 nucleotides downstream of the translation stop codon of slt-IIB.

Table 5. Oligonucleotides used in this study

Name	Sequence <sup>a</sup>	Position <sup>b</sup>	Purpose
<b>Primer extensions</b>			
PEI1	5'-AAACCCAGTAACAGGCACAGTACC-3'	<u>slt-IIA</u> : +47 to +24	<u>slt-II</u> promoter search
PEI3	5'-ATAATATACACTTCATATACAGGTG-3'	<u>slt-IIA</u> : +16 to -9	<u>slt-II</u> promoter search
PEI4	5'-GTAATCAGTACCAGACCCGGCGCA-3'	<u>slt-IIVA</u> : +48 to +25	<u>slt-IIv</u> promoter search
PEI5	5'-CGCCATTGCATTAACAGAAAGC-3'	<u>slt-IIIB</u> : +57 to +37	Independent <u>slt-IIB</u> promoter search
PEI6	5'-TGTCATCCTCATTATACTTGG-3'	<u>slt-IIIB</u> : +109 to +89	Independent <u>slt-IIB</u> promoter search
<b>Site-specific mutagenesis</b>			
RECHP1	5'-CGGCTGAGTTAACAACGCATAATGC-3'	<u>slt-IIA</u> : -150 to -174	Creation of <u>HpaI</u> site in <u>slt-II</u>
RECBH1	5'-TGTA <del>CTGGATCC</del> CGAACCCAGTGACCCG-3'	<u>slt-IIA</u> : -99 to -123	Creation of <u>Bam</u> HI site in <u>slt-II</u>
STX <del>BH1</del>	5'-AATACTCCTTGGGATCCATACGAT-3'	<u>stxA</u> : -12 to -34	Creation of <u>Bam</u> HI site in <u>stx</u>
<b>Sequencing primers</b>			
S11I2	5'-GGGAATAGGATACCGAAGAAAACCC-3'	<u>slt-IIA</u> : +67 to +42	S1 nuclease sequence ladder
SLT1I9	5'-GTGGGGCGACTGGTG-3'	<u>slt-IIIB</u> : +124 to +139	Sequence data ( <u>slt-II</u> terminator)

15 mM Tris pH 8.0, 0.45 M sucrose, and 8 mM EDTA. The pellet was then mixed with 80  $\mu$ l of 50 mg/ml lysozyme, 100  $\mu$ l of 100 mM dithiothreitol (DTT), and 10,000 units of RNasin. This suspension was incubated on ice for 15 minutes and then subjected to centrifugation. Cells were resuspended in 3.5 ml of 4 M guanidinium isothiocyanate, 20 mM sodium acetate pH 5.2, 0.1 mM DTT, and 0.5% N-lauryl sarcosine. The solution was heated for 30 minutes at 65°C and then drawn through a 20 gauge needle several times. The cell lysate was layered onto 7 ml of 5.7 M cesium chloride and subjected to centrifugation at 30,000 rpm in a Beckman SW40.1 rotor at 20°C for 12 hours. The RNA pellet was resuspended in water, dispensed into aliquots, and each aliquot was precipitated with ethanol.

**Primer extension analysis.** Total cellular RNA (50  $\mu$ g) and 25 ng of a  $^{32}$ P 5'-end labeled oligonucleotide primer were co-precipitated and resuspended in 30  $\mu$ l of 40 mM PIPES pH 6.7, 1 mM EDTA, 0.2% sodium dodecyl sulfate, and 0.4 M sodium chloride. The mixture was heated to 80°C for 5 minutes and then at 37°C for 2 hours to permit the annealing of the oligonucleotide primer to the RNA. This RNA/primer complex was then precipitated with ethanol, and the precipitate was resuspended in 50  $\mu$ l of the following solution: 50 mM Tris-HCl pH 8.3, 6 mM magnesium chloride, 40 mM potassium chloride, and 10 mM DTT, 2.5  $\mu$ l each of 10 mM dNTP (dATP, dGTP, dCTP, and dTTP), and 20 units of Maloney-murine

leukemia virus reverse transcriptase. Extension of the oligonucleotide primer to the 5' terminus of the RNA was allowed to proceed for 2 hours. The RNA/cDNA complex was then treated with 1  $\mu$ l of a 10 mg/ml RNase A solution, extracted with phenol/chloroform, and precipitated with ethanol. The pellet was resuspended in 5  $\mu$ l of formamide loading buffer and subject to polyacrylamide gel electrophoresis (PAGE) on a vertical sequencing gel apparatus. Dideoxy chain termination sequencing reaction mixtures were used as nucleotide length markers.

To map the promoter upstream of slt-IIA, the noncoding sequence ladder was generated using the coding strand of the 1100 base pair SphI-EcoRV fragment of pNN103 in M13 mp19 as the template and either oligonucleotide PEII1 or PEII3 (Table 5) as the primer. To map the promoter upstream of slt-IIvA, the noncoding sequence ladder was generated using the coding strand of the 4200 base pair AatII-ClaI fragment of pDLW5 in M13 mp19 as the template and oligonucleotide PEIV4 (Table 5) as the primer. To attempt to locate an independent slt-IIB promoter, the noncoding sequence ladder was generated with the coding strand of the 772 base pair EcoRV-PstI fragment of pNN103 in M13 mp19 as the template and either oligonucleotide PEIIB5 or PEIIB6 (Table 5) as the primer.

**S1 nuclease protection analysis.** Total cellular RNA (50  $\mu$ g) from E. coli HB101(pNN103) and 50 ng of a  $^{32}$ P 5'-end labeled 1030 bp SphI-SmaI fragment

of pNN103 were co-precipitated and resuspended in 20  $\mu$ l of 40 mM PIPES pH 6.8, 90% formamide, 0.4 M sodium chloride, and 1 mM EDTA. The mixture was heated at 90°C for 10 minutes, and the RNA and DNA fragment were permitted to anneal at 49°C for 12 hours. The RNA/DNA complex was then mixed with 200  $\mu$ l of 0.25 M sodium chloride, 30 mM sodium acetate pH 4.5, and 1 mM zinc sulfate, followed by 10 units of S1 nuclease. This mixture was then incubated for 30 minutes. The RNA/DNA complex was then treated with 1  $\mu$ l of a 10 mg/ml RNase A solution, extracted with phenol/chloroform, and precipitated with ethanol. The pellet was resuspended in 5  $\mu$ l of formamide loading buffer and subject to polyacrylamide gel electrophoresis (PAGE) on a vertical sequencing gel apparatus. Dideoxy chain termination sequencing reaction mixtures were used as nucleotide length markers. This ladder represents the noncoding strand sequence and was generated using the coding strand of the 1100 base pair SphI-EcoRV fragment of pNN103 in M13 mp19 as the template and the oligonucleotide S1II2 (Table 5) as the primer.

**Confirmation of the slt-II operon terminator.** The nucleotide sequence of regions downstream of the slt-IIB open reading frame was examined by computer homology search using the Genetics Computer Group (University of Wisconsin, Madison, WI) sequence analysis software package. The computer program, Terminator, was used to search for prokaryotic factor independent RNA poly-

merase terminators according to the algorithm of Brendel and Trifonov, 1984. The 50 base pair StuI-NruI fragment of pNN103, which contained the putative slt-II transcription terminator, was inserted into pLMS4.0 (Table 4) between the lac promoter and the translation start codon of the chloramphenicol acetyltransferase gene (cat) to create pLMS4.4 (Table 4). The activity of this construct was determined by CAT enzyme-linked immunosorbent assay (ELISA).

**Northern blot analysis.** Total cellular RNA (50  $\mu\text{g}$ ) from E. coli HB101 (pNN103) was dissolved in 22.5  $\mu\text{l}$  dimethyl sulfoxide (DMSO), 4.5  $\mu\text{l}$  sodium phosphate pH 7, and 6.6  $\mu\text{l}$  glyoxal. This solution was incubated at 37°C for 1 hour, cooled on ice, and 12  $\mu\text{l}$  of glyoxal loading buffer was added. The samples were subjected to electrophoresis in a 1.2% agarose gel at 4 volts per centimeter with constant buffer recirculation. Ribosomal RNAs (23S, 16S, and 5S) were run alongside the samples as size standards. The RNA was transferred onto a nitrocellulose filter using a Vacu-Blot apparatus (Pharmacia LKB Systems). The filter was baked at 80°C for 2 hours in a vacuum oven and soaked in a pre-hybridization solution (Selden et al., 1989) for at least 2 hours. Filters were probed with either a 460 base pair HpaI-KpnI fragment of pMJ330 (Table 4) which contains only slt-IIB subunit coding sequences or a 1350 base pair SmaI-KpnI fragment of pMJ330 which contains both slt-IIA and slt-IIB coding sequences.

**Oligonucleotide-directed, site-specific mutagenesis.** To create restriction sites in the SLT-II operon, a M13 mp19 vector containing the 1100 base pair SphI-EcoRV fragment of pNN103 (Table 4) was propagated in E. coli CJ236 by using the procedure provided by the supplier of the Muta-Gene DNA mutagenesis kit (Bio-Rad Laboratories). The single-stranded DNA template was annealed to RECHP1 (Table 5), an oligonucleotide designed to create a HpaI site 162 nucleotides upstream of slt-IIA. The resulting single-stranded template which now contained the HpaI site was propagated in E. coli CJ236 as described above and subsequently used with the mutagenic oligonucleotide RECBH1 (Table 5) to introduce a BamHI site 110 nucleotides upstream of slt-IIA. A 1030 base pair SphI-SmaI fragment containing both newly introduced sites was ligated with the 2200 base pair SmaI-EcoRI fragment of pNN103 into vector pBR329 to construct pLMS1.0 (Table 4, Figure 9).

To create a restriction site between the promoter and structural genes in the Shiga toxin operon, a M13 mp19 vector containing the 725 base pair BglII-HindIII fragment of pNAS13 was propagated in E. coli CJ236. The single-stranded DNA template was annealed to STXBH1 (Table 5), an oligonucleotide designed to create a BamHI site 24 nucleotides upstream of stxA. A 746 base pair EcoRI-HindIII fragment containing the newly introduced site was ligated to the 1800 base pair HindIII-EcoRI fragment of pNAS13 into vector pBR329 to construct pLMS0.0 (Table 4).

**Creation of promoter-deletion constructs.** Shiga-like toxin promoter-deletion subclones were constructed as depicted in Figure 10. The 2445 base pair HpaI-EcoRI fragment of pLMS1.0 was cloned into vector pBluescript KS to construct pLMS2.1 (Table 4). The 2395 base pair BamHI-EcoRI fragment of pLMS1.0 was cloned into vector pBluescript KS to construct pLMS2.2 (Table 4). The 3800 base pair EcoRI-ClaI fragment of pDLW5.125 was cloned into vector pBluescript KS to construct pLMS2.3 (Table 4). Each fragment was inserted into pBluescript KS in an orientation opposite to the direction of transcription from known vector promoters.

**Creation of Shiga-like toxin promoter / CAT transcriptional fusions.**

The 139 base pair BglII-BamHI fragment of pLMS0.0, which contains the stx promoter, was cloned into the promoter analysis vector pKK232-8 to create pLMSCATI (Table 4, Figure 11). The 51 base pair HpaI-BamHI fragment of pLMS1.0, which contains the slt-II promoter, was cloned into pKK232-8 to create pLMSCATII (Table 4, Figure 11). The 216 base pair PvuII-HindIII fragment of pBS, which contains the lac promoter, was cloned into pKK232-8 to create pLMSCATC (Table 4, Figure 11). The activities of these constructs were determined by CAT enzyme ELISA.

**Creation of promoter / structural gene chimeras.** The 162 base pair EcoRI-BamHI fragment of pLMS0.0, which contains the stx promoter, was ligated with the 2395 base pair fragment of pLMS1.0, which contains the structural genes for SLT-II, in vector pBR329 to create pLMS100 (Table 4, Figure 12). The 854 base pair SphI-BamHI fragment of pLMS1.0, which contains the slt-II promoter, was ligated with the 2384 base pair fragment of pLMS0.0, which contains the structural genes for Shiga toxin, in vector pBR329 to create pLMS200 (Table 4, Figure 12).

**Mouse colonization experiments.** Mice were colonized with various E. coli strains according to the method of Myhal et al., 1982 and 1983, with the following modifications. Five to eight week old CD-1 male mice (Charles River Laboratories, Wilmington, MA) were given drinking water containing streptomycin sulfate (5 grams per liter) and ampicillin (5 grams per liter) to reduce the normal intestinal facultative bacterial flora of the mice. After 1 day of antibiotic treatment, the number of facultative bacteria dropped from about  $10^8$  to less than  $10^2$  colony forming units (CFU) per gram of feces, and the number of anaerobic bacteria remained constant at about  $10^9$  CFU per gram of feces. Food and water were then withheld from the mice for 18 to 24 hours. Groups of three mice were then fed by calibrated droppers approximately  $10^{10}$  CFU of each E. coli strain to be tested. The mice readily ingested the bacterial suspension which was prepared

by harvesting the bacteria by centrifugation (7,500 rpm for 5 min.), washing the bacterial pellet twice in phosphate buffered saline (PBS), pH 7.4, and suspending the washed bacteria in 1 ml of sterile 20% (w/v) sucrose. The animals were then housed individually and permitted food and water containing 5 grams per liter streptomycin sulfate and ampicillin ad libitum. The following day and at 24 or 48 hours intervals as indicated, 1 g of feces was collected, homogenized in 10 ml of 1% tryptone, and 10-fold serial dilutions of the homogenate prepared and plated on agar media that contained the appropriate antibiotics. The plates were incubated at 37°C for 18 to 24 hours. The degree to which a given strain colonized was determined by the number of CFU that persisted in the feces. Each colonization experiment was performed at least three times.

## RESULTS

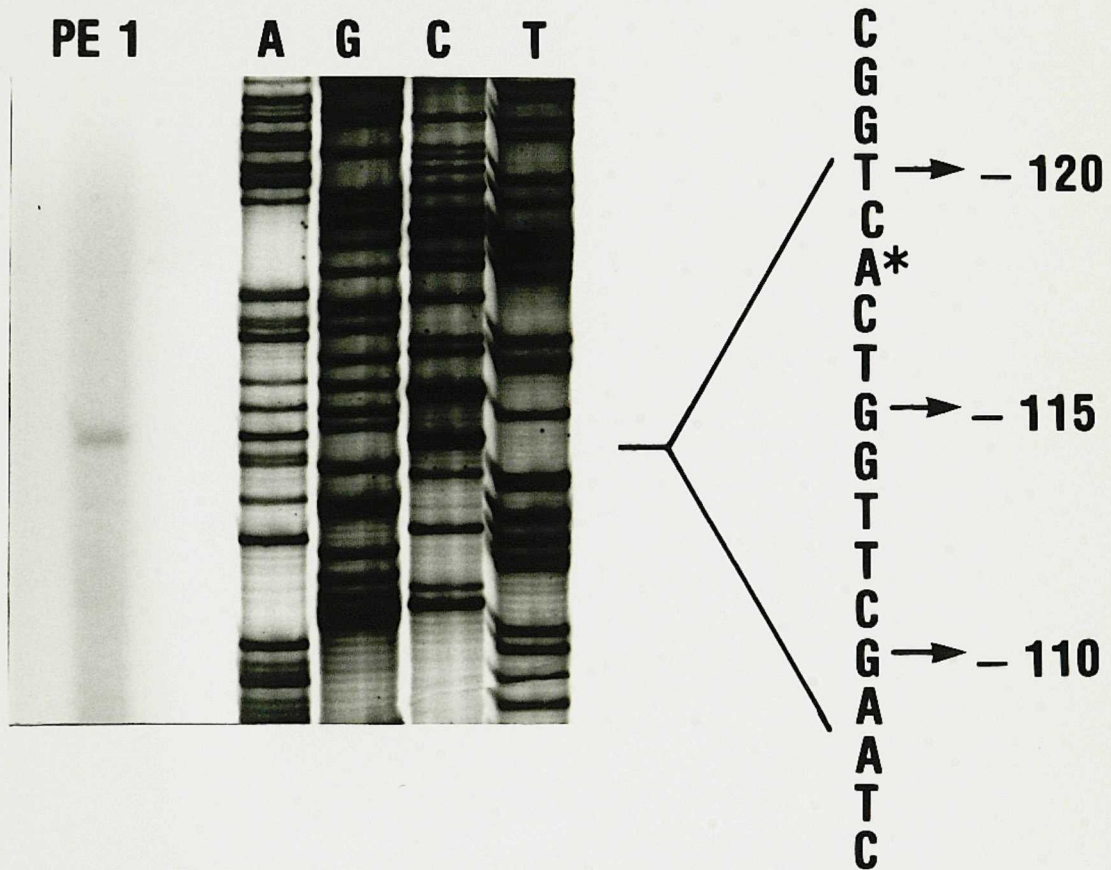
### I. Characterization of the SLT-II operon.

**Promoter mapping of the SLT-II and SLT-IIv operons.** To identify the transcription start site for the SLT-II operon, primer extension experiments were done with oligonucleotide primer PEII1 (Table 5) using total cellular RNA isolated from either the SLT-II clone *E. coli* HB101(pNN103) (Figure 1), or the SLT-II phage lysogen *E. coli* C600(933W) (data not shown). In both cases, a band was observed that comigrated with chains terminated at a specific thymine in the sequence ladder. This band represented an extension of 142 bases to a position located 118 nucleotides upstream of the slt-IIA initiation codon and corresponded to an adenine residue in the coding strand. No bands were observed with control primer extension reactions using total cellular RNA from an *E. coli* HB101 strain.

The location of the slt-II transcription start site was independently established using a second oligonucleotide primer, PEII3 (Table 5), in additional primer extension experiments with total cellular RNA from *E. coli* HB101(pNN103) (Figure 2). A transcript of 109 bases was observed, which reflects an extension to the same nucleotide position upstream of slt-IIA that was previously identified. Therefore, primer extensions from two independent positions within the slt-IIA gene identified the adenine residue 118 nucleotides upstream of the slt-IIA open reading

### Figure 1 Legend

**Primer extension analysis.** Total cellular RNA isolated from E. coli HB101(pNN103) was probed with oligonucleotide primer PEII1 (Table 5). The dideoxy sequence ladder represents the noncoding strand sequence and was generated with the coding strand of the 1100 base pair SphI-EcoRV fragment of pNN103 (Table 4) in M13 mp19 as the template and PEII1 as the oligonucleotide primer. A band comigrated with chains terminated at a thymine that corresponds to an adenine residue on the coding strand located 118 nucleotides upstream of the slt-IIA initiation codon.

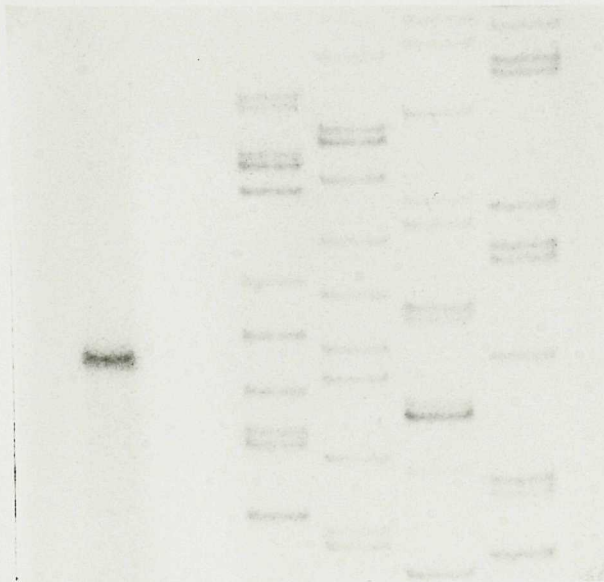


### Figure 2 Legend

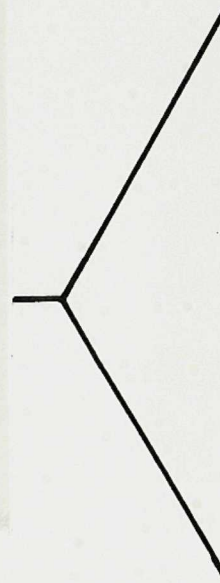
**Primer extension analysis.** Total cellular RNA isolated from E. coli HB101(pNN103) was probed with oligonucleotide primer PEII3 (Table 5). The dideoxy sequence ladder represents the noncoding strand sequence and was generated with the coding strand of the 1100 base pair SphI-EcoRV fragment of pNN103 (Table 4) in M13 mp19 as the template and PEII3 as the oligonucleotide primer. A band comigrated with chains terminated at a thymine that corresponds to an adenine residue on the coding strand located 118 nucleotides upstream of the slt-IIA initiation codon.

PE 3

A G C T



C  
G  
G  
T → - 120  
C  
A\*  
C  
T  
G → - 115  
G  
T  
T  
C  
G → - 110  
A  
A  
T  
C



frame on the coding strand as the transcription start site.

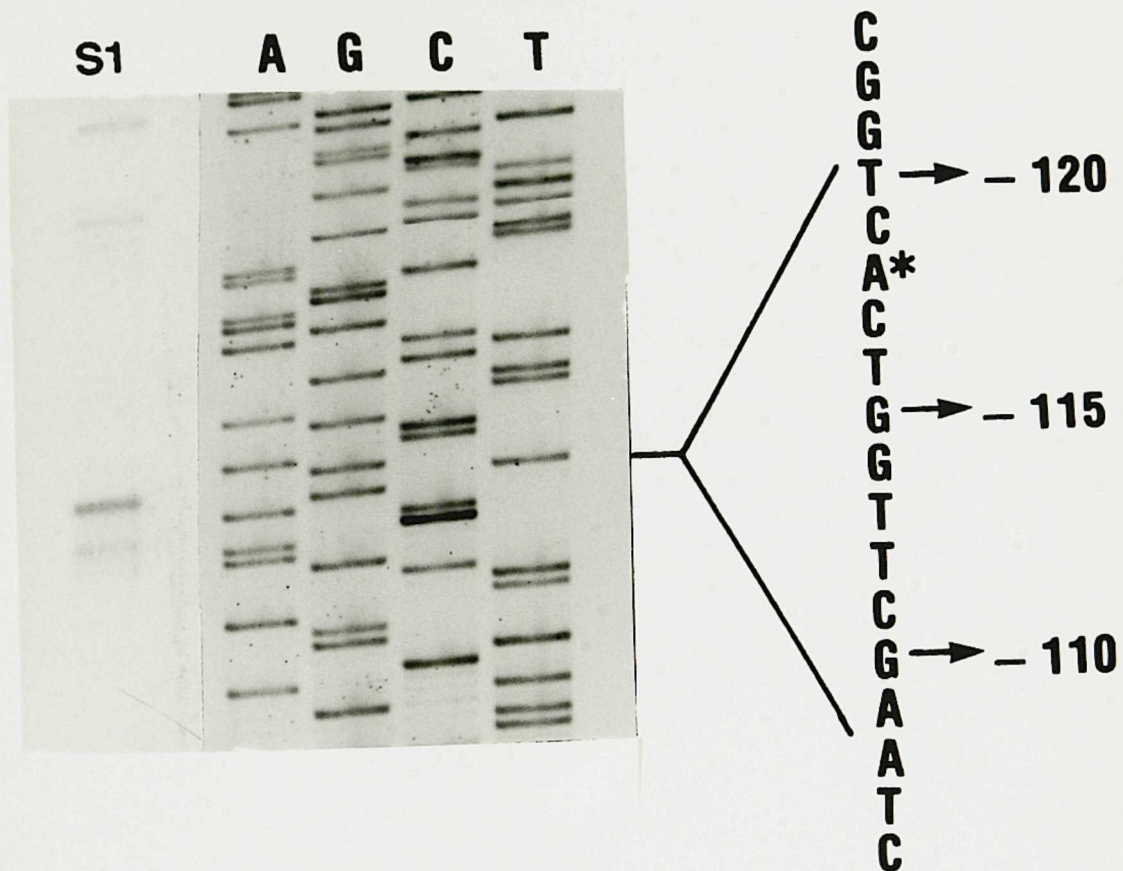
Primer extension analysis was also used to search for a possible second promoter for the transcription of the B subunit. Two oligonucleotides, PEIIB5 and PEIIB6 (Table 5), designed to anneal at different positions within slt-IIB were used. No transcription start sites could be identified in these primer extension experiments. These experiments provide no evidence that the independent transcription of the B subunit occurs in the SLT-II operon.

As a second method of identifying the transcription start site for the SLT-II operon, RNA transcripts from E. coli HB101(pNN103) were examined by S1 nuclease protection studies. A 185 base pair fragment protected from S1 nuclease digestion comigrated with chains terminated at a thymine in the sequence ladder (Figure 3), which again corresponds to the adenine residue 118 nucleotides upstream of the slt-IIA open reading frame on the coding strand.

Because of the high degree of nucleotide sequence homology between slt-II and slt-IIv the possibility that the two operons have the same promoter was tested. Total cellular RNA isolated from the SLT-IIv clone, E. coli HB101(pDLW5) and the oligonucleotide PEIIV4 (Table 5) were used in a primer extension analysis to identify the slt-IIv promoter and compare it with the putative slt-II promoter. A transcription start site was identified at an adenine residue 119 nucleotides upstream of the slt-IIvA initiation codon (data not shown). Alignment of the nucleotide sequences upstream of slt-IIA and slt-IIvA revealed the addition of a

### Figure 3 Legend

**S1 nuclease protection analysis.** Total cellular RNA isolated from *E. coli* HB101(pNN103) was probed with the 1030 base pair SphI-SmaI fragment of pNN103 (Table 4) which includes the first 67 nucleotides of slt-IIA and sequences upstream. The dideoxy sequence ladder represents the noncoding strand sequence and was generated with the coding strand of the 1100 base pair SphI-EcoRV fragment of pNN103 in M13 mp19 as the template and S112 as the oligonucleotide primer (Table 5). A band comigrated with chains terminated at a thymine that corresponds to an adenine residue on the coding strand located 118 nucleotides upstream of the slt-IIA initiation codon.



### Figure 4 Legend

Previously determined nucleotide sequences for stx/slt-I, slt-II, and slt-IIv are shown aligned with respect to the initiation codon for the A subunit. A dash (-) represents a nucleotide identical to the one at that position in slt-II. A single base addition (+) was found at position -91 of slt-IIv. The -35 and -10 promoter sequences for each operon are underlined as are the -118 adenine transcription start site for the SLT-II operon and the novel -60 transcription start site identified in the promoter deletion subclone pLMS2.2. Newly created restriction endonuclease sites are depicted as follows: ○, BglII site in stx; ⊖, created HpaI site in slt-II; ⊙, created BamHI site in slt-II; ⊗, created EcoRI site in slt-IIv; and ⊕, created BamHI site in stx.



single base at position -91 of slt-IIv (Figure 4). Therefore, the -119 adenine of slt-IIv is homologous to the -118 adenine of slt-II. A further sequence comparison indicated that slt-II and slt-IIv differ by only two nucleotides in this region (Figure 4). Based on the transcription start sites identified, the putative -35 and -10 promoter sequences for slt-II and slt-IIv are, as predicted, identical.

The -35 and -10 sequences of the putative slt-II/slt-IIv promoter are not homologous to those of the stx/slt-I promoter, nor do they conform well to the consensus -35 and -10 sequences established from a compilation of known E. coli promoters. In general, promoters which lack homology with the consensus sequence are less efficient at initiating transcription than promoters with a high degree of homology (Rosenberg and Court, 1979, and Hawley and McClure, 1983). Also in contrast to the stx/slt-I promoter, no Fur repressor binding site was found in the putative slt-II/slt-IIv promoter region.

**Terminator mapping of the SLT-II operon.** A computer search for sequence homology with a consensus sequence derived from known E. coli terminators revealed a potential rho-independent transcription termination sequence 274 base pairs downstream of slt-IIb. To confirm that the predicted sequence functions as a transcription terminator, the putative terminator sequence was inserted into pLMS4.0 (Table 4, Figure 5) between the lac promoter and the chloramphenicol acetyltransferase (cat) gene. The resultant construct, pLMS4.4

### Figure 5 Legend

**Transcription terminator verification.** The 50 base pair StuI-NruI fragment of pNN103, which contained the predicted slt-II transcription terminator sequence, was inserted into pLMS4.0 between the lac promoter and the chloramphenicol acetyltransferase (cat) gene to create pLMS4.4. Arrows indicate the direction of transcription of the cat gene from the lac promoter.



**pLMS4.0**



**pLMS4.4**

*slt-II* transcription terminator

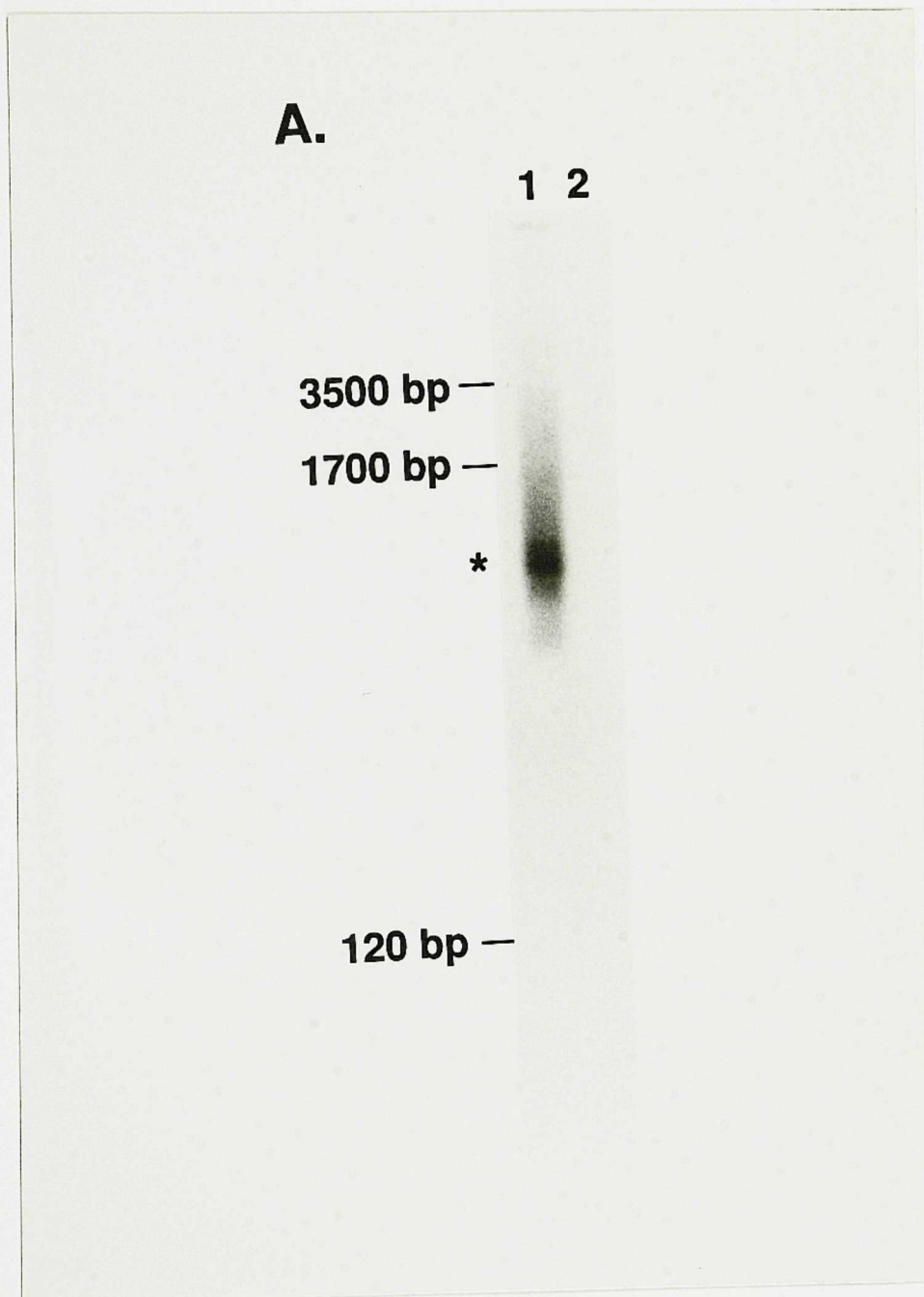
(Table 4, Figure 5), produced 100-fold less CAT protein than pLMS4.0. This finding demonstrated the functional activity of the mapped slt-II terminator.

**Transcriptional analyses of the SLT-II operon.** Transcripts of the SLT-II operon were examined by Northern blot analysis. Filters were probed with a DNA fragment containing both slt-IIA and slt-IIB sequences. A single band was observed (Figure 6) which corresponded to the expected size of a transcript proceeding from the putative slt-II promoter to the terminator. When the same blots were probed with a smaller DNA probe containing only slt-IIB sequences, the same band was observed (Figure 7). With neither probe were other bands apparent, even after prolonged autoradiograph exposure. The results reported here of Northern blot and primer extension analyses of slt-II clearly indicated that transcription of the SLT-II operon occurs as a single unit and provide no evidence for an independent promoter for the slt-IIB gene.

**Iron-regulation of SLT-II.** The expression of prophage-encoded slt-I and slt-II genes was compared in several E. coli strains that differed with respect to the presence or absence of the fur allele and its copy number (Figure 8). The SLT-I converting phage H19J or the SLT-II converting phage 933W was lysogenized into three different E. coli strains: AB2847, which contains the chromosomal fur<sup>+</sup> allele; H1618, a fur<sup>-</sup> mutant of AB2847; and H1646, constructed by introduc-

### Figure 6 Legend

**Northern blot analysis.** Total cellular RNA was denatured with glyoxal / DMSO, subjected to electrophoresis on a 1.4% agarose gel, and transferred to nitrocellulose. Lane 1 and Lane 2 represent total cellular RNA isolated from E. coli HB101(pNN103) and an E. coli HB101(pBR329) control, respectively. The filter was probed with a 1350 base pair SmaI-KpnI fragment of pMJ330 (Table 4) which contains both slt-IIA and slt-IIB coding sequences.



### Figure 7 Legend

**Northern blot analysis.** Total cellular RNA was denatured with glyoxal / DMSO, subjected to electrophoresis on a 1.4% agarose gel, and transferred to nitrocellulose. Lane 1 and Lane 2 represent total cellular RNA isolated from E. coli HB101(pNN103) and an E. coli HB101(pBR329) control, respectively. The filter was probed with a 460 base pair HpaI-KpnI fragment of pMJ330 (Table 4) which contains only slt-IIB subunit coding sequences.

**B.**

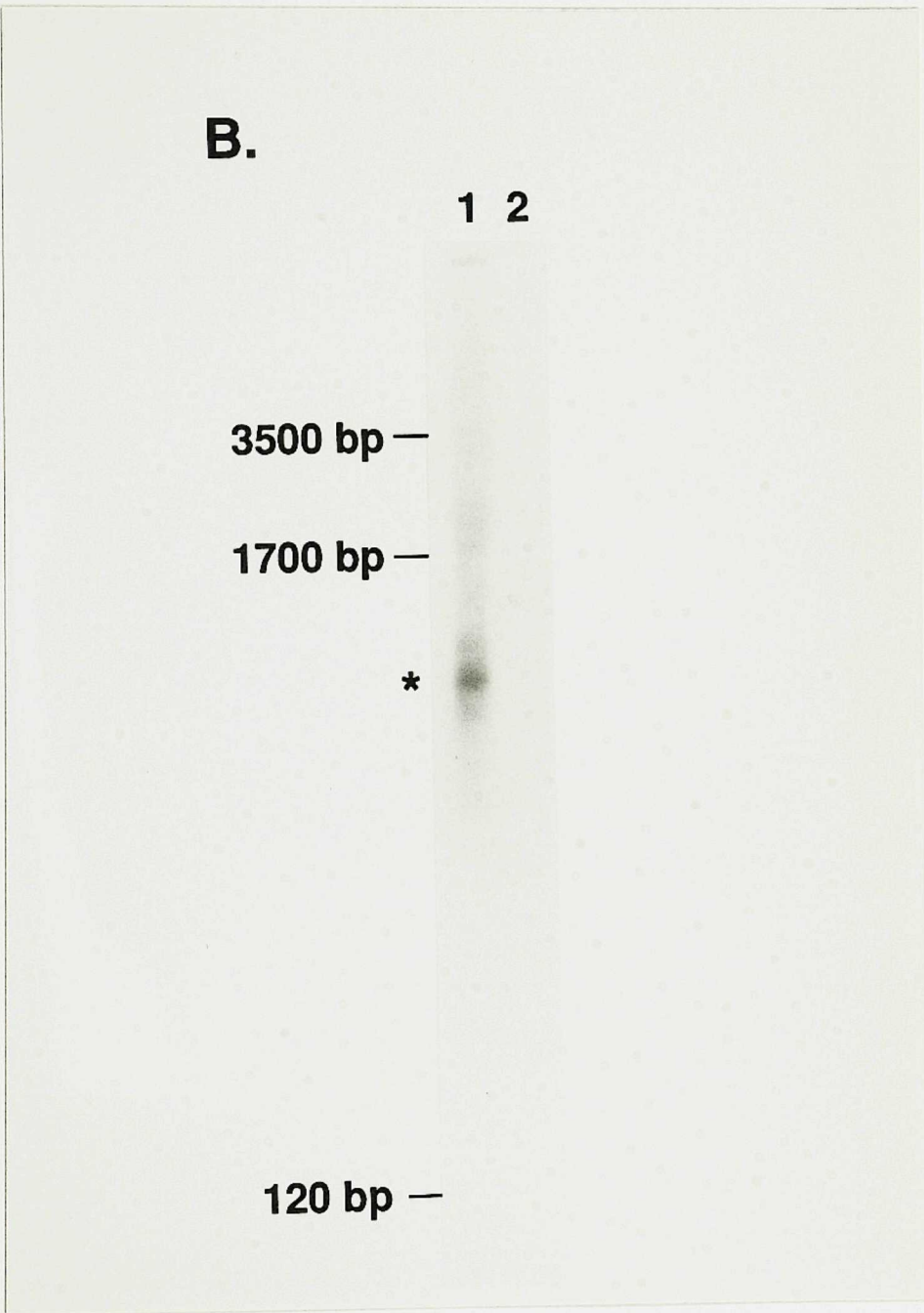
**1 2**

**3500 bp —**

**1700 bp —**

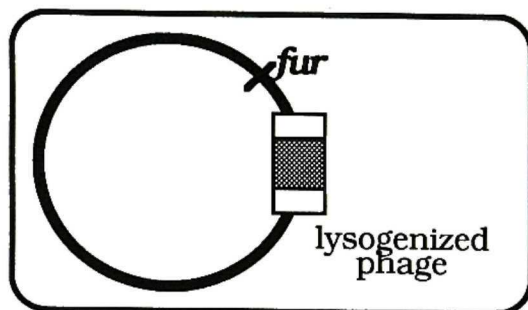
**\***

**120 bp —**

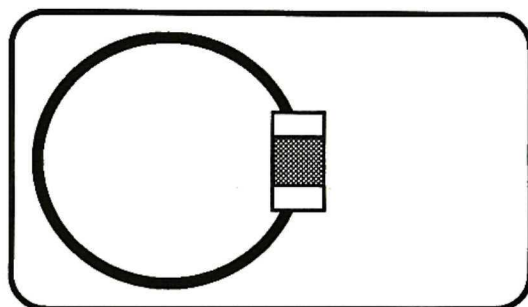


### Figure 8 Legend

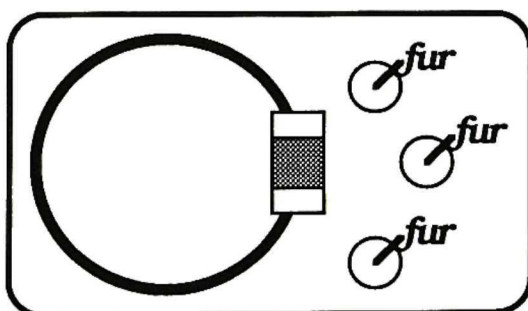
**Strain construction.** Several *E. coli* strains that differed with respect to the presence or absence of the *fur* allele and its copy number were lysogenized with either the SLT-I or SLT-II converting phage. A, Wild-type *E. coli* AB2847 lysogen; B, *E. coli* H1618 lysogen, *fur*<sup>-</sup> mutant of AB2847; and C, *E. coli* H1646 lysogen, constructed by introducing the high copy number *fur* plasmid pMH1 into strain H1618.

**A**

■ single copy toxin  
single copy *fur*

**B**

■ single copy toxin  
no *fur*

**C**

■ single copy toxin  
multiple copies of *fur*

ing the high copy number fur plasmid pMH1 into strain H1618. In each of these strains, the expression of the prophage-encoded slt-I or slt-II was compared under low-iron and high-iron growth conditions (Table 6). SLT-I production was iron-regulated in the H19J fur<sup>+</sup> lysogens AB2847(H19J) and H1646(H19J), but not in the H19J fur<sup>-</sup> lysogen H1618(H19J), confirming previous reports on iron repression of SLT-I production. Under identical conditions, SLT-II production, as seen in 933W lysogens AB2847(933W), H1618(933W), and H1646(933W), was independent of the iron concentration and the copy number of the fur gene, although toxin levels were several hundred-fold greater in the AB2847 background than in H1618 or H1646. These findings confirm and extend the conclusion that the fur gene product plays no role in the regulation of the SLT-II operon.

**SLT-II production in E. coli crp mutant backgrounds.** To determine if the slt-II operon was positively regulated by the cyclic AMP regulatory protein (CRP), the expression of plasmid-encoded slt-II genes was compared in two E. coli strains, JK7 and JK7(pHA5), that differed with respect to the presence or absence of the gene which codes for CRP (crp). No difference in the levels of SLT-II was observed in these backgrounds (data not shown). This indicated that the CRP has no involvement with the expression of the SLT-II operon.

**Table 6 Legend**

- a Four samples tested per group.
- b Symbols: (+), single chromosomal fur copy; (+++), multiple plasmid-encoded fur copies; and (-), point mutation in chromosomal fur copy, fur<sup>-</sup> phenotype.
- c Cell-associated,  $\log_{10}$  50 % cytotoxic dose ( $CD_{50}$ ) per pellet; Extracellular,  $\log_{10}$   $CD_{50}$  per 50 ml of supernatant; Total/ $A_{600}$ ,  $\log_{10}$  cell-associated  $CD_{50}$  plus  $\log_{10}$  extracellular  $CD_{50}$  divided by absorbance at 600 nm. Mean value  $\pm$  2 standard errors of the mean.
- d Significantly different ( $p < 0.05$ ) by Student's unpaired t test from value for the same lysogen grown in the absence of added iron.

Table 6. Effects of iron on SLT-II production

E. coli lysogen <sup>a</sup>	Toxin	fur gene <sup>b</sup>	Iron	Amount of cytotoxin (mean $\pm$ 2 SEM) <sup>c</sup>		
				Cell-associated	Extracellular	Total/A <sub>600</sub>
AB2847(H19J)	SLT-I	+	+	7.5 $\pm$ 0.2 <sup>d</sup>	8.6 $\pm$ 0.3	7.5 $\pm$ 0.4 <sup>d</sup>
H1618(H19J)	SLT-I	-	-	8.6 $\pm$ 0.2	8.6 $\pm$ 0.2	8.5 $\pm$ 0.3
H1646(H19J)	SLT-I	-	+	6.5 $\pm$ 0.3	6.8 $\pm$ 0.3	6.9 $\pm$ 0.4
H1646(H19J)	SLT-I	+++	-	6.8 $\pm$ 0.2	7.0 $\pm$ 0.3	7.1 $\pm$ 0.3
AB2847(933W)	SLT-II	+	+	7.0 $\pm$ 0.3 <sup>d</sup>	6.5 $\pm$ 0.3	6.6 $\pm$ 0.3 <sup>d</sup>
H1618(933W)	SLT-II	-	-	8.4 $\pm$ 0.1	6.8 $\pm$ 0.2	8.1 $\pm$ 0.2
H1646(933W)	SLT-II	+++	+	5.4 $\pm$ 0.2	6.5 $\pm$ 0.7	5.9 $\pm$ 0.5
H1646(933W)	SLT-II	-	-	5.2 $\pm$ 0.1	6.5 $\pm$ 0.6	5.9 $\pm$ 0.4
H1646(933W)	SLT-II	-	+	3.1 $\pm$ 0.1	3.6 $\pm$ 0.2	3.6 $\pm$ 0.2
H1646(933W)	SLT-II	+++	-	3.1 $\pm$ 0.1	3.9 $\pm$ 0.3	3.7 $\pm$ 0.3
H1646(933W)	SLT-II	+++	+	3.2 $\pm$ 0.2	3.1 $\pm$ 0.3	3.0 $\pm$ 0.2
H1646(933W)	SLT-II	-	-	3.1 $\pm$ 0.1	3.2 $\pm$ 0.1	3.0 $\pm$ 0.1

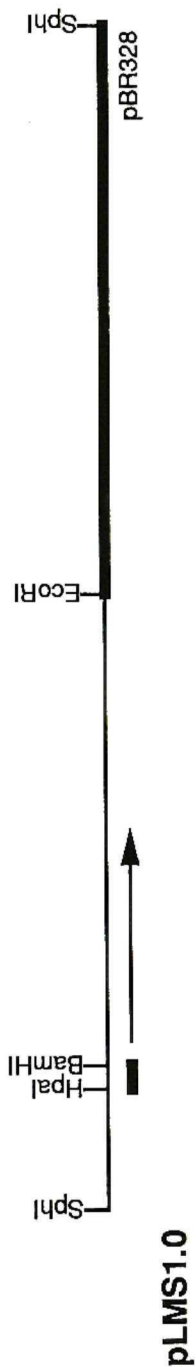
## II. Functional analysis of the slt-II/slt-IIv promoter.

Oligonucleotide-directed, site-specific mutagenesis was used to create HpaI and BamHI restriction endonuclease sites bracketing the putative slt-II promoter sequences in plasmid pLMS1.0 (Table 4, Figure 9), as well as a BamHI site downstream of the stx promoter in plasmid pLMS0.0 (Table 4). These new restriction sites permitted the isolation of the promoter sequences for an examination of transcriptional activity.

**Functional confirmation of the slt-II and slt-IIv promoters.** Promoter-deletion subclones were analyzed to establish that the putative promoter sequences mapped by primer extension and S1 nuclease protection studies were indeed the functional promoters for the SLT-II and SLT-IIv operons. Sequences adjacent to the HpaI site upstream of the putative slt-II promoter were deleted in pLMS2.1 (Figure 10). As shown in Table 7, *E. coli* DH5 $\alpha$ (pLMS2.1) produced  $10^3$  50% cytotoxic doses per ml ( $CD_{50}$ /ml) of SLT-II. Next, the HpaI-BamHI fragment containing the putative slt-II promoter was also deleted during the construction of pLMS2.2 (Figure 10). Surprisingly, *E. coli* DH5 $\alpha$ (pLMS2.2) was also cytotoxic at  $10^3$   $CD_{50}$ /ml. Primer extension analysis performed on total RNA isolated from the promoter-deletion subclone pLMS2.2 revealed a new transcription start site 60 nucleotides upstream of the slt-IIA open reading frame (Figure 4). Cytotoxin production by *E. coli* DH5 $\alpha$ (pLMS2.2) was therefore attributed to transcription of

### Figure 9 Legend

**Restriction endonuclease site creation.** Oligonucleotide-directed site-specific mutagenesis was used to create HpaI and BamHI restriction endonuclease sites bracketing the putative slt-II promoter sequences. The construct pLMS1.0 contains these newly created sites and is a subclone of pNN103, which carries the entire SLT-II operon. Arrows indicate the location of the toxin structural genes and the direction of transcription from the respective promoter (indicated by a solid block located upstream of the coding region).



### Figure 10 Legend

**Shiga-like toxin promoter deletion subclone strategy.** Recombinant plasmids pLMS2.1 and pLMS2.2 were created as subclones of pLMS1.0. Recombinant plasmid pLMS2.3 was created as a subclone of pDLW5.125. Arrows indicate the location of the toxin structural genes and the direction of transcription from the respective promoter (indicated by a solid block located upstream of the coding region). Vector DNA is represented by bold lines.



**Table 7. Cytotoxicity of promoter deletion subclones in *E. coli* DH5 $\alpha$** 

Plasmid construct <sup>a</sup>	Toxin operon	Promoter	Cytotoxicity <sup>b</sup>
pNN103	SLT-II	+	10 <sup>4</sup>
pLMS1.0	SLT-II	+	10 <sup>4</sup>
pLMS2.1	SLT-II	+	10 <sup>3</sup>
pLMS2.2	SLT-II	-	10 <sup>3</sup>
pDLW5	SLT-IIv	+	10 <sup>3</sup>
pDLW5.125	SLT-IIv	+	10 <sup>3</sup>
pLMS2.3	SLT-IIv	-	BLD <sup>c</sup>

<sup>a</sup> As described in Table 4. Four samples tested per group.

<sup>b</sup> Log<sub>10</sub> 50% cytotoxic dose (CD<sub>50</sub>) per ml of sonically disrupted *E. coli* DH5 $\alpha$  transformed with the plasmid construct. Cytotoxicity represents CD<sub>50</sub> per ml observed for all four samples tested.

<sup>c</sup> Below the limit of detection, less than 10<sup>1</sup> CD<sub>50</sub>.

the SLT-II genes from a secondary promoter which was inactive in the wild-type SLT-II operon. Direct confirmation that the HpaI-BamHI fragment contained a functional promoter was provided by the operon fusion experiments described in the next section.

The putative slt-IIv promoter located within the AatII-EcoRI fragment of pDLW5.125 was deleted in the construction of pLMS2.3 (Figure 10). No detectable toxin activity (less than  $10^1$  CD<sub>50</sub>/ml) was observed with E. coli DH5 $\alpha$  (pLMS2.3) (Table 7). In contrast, E. coli DH5 $\alpha$  harboring pDLW5.125 (Figure 10), which retained the putative slt-IIv promoter sequences, was cytotoxic at  $10^3$  CD<sub>50</sub>/ml. These data support the assignment of the slt-IIv promoter based on nucleotide sequence and primer extension analysis.

**Comparison of transcriptional efficiencies of the stx and slt-II promoters.** The transcriptional activities of the stx and the slt-II promoters were examined using the promoter analysis vector pKK232-8 in which the expression of the chloramphenicol acetyltransferase (cat) gene was regulated by the heterologous promoter fragment inserted (Figure 11). The amounts of CAT enzyme produced by the fur<sup>+</sup> strain E. coli DH5 $\alpha$  harboring the stx promoter / cat fusion pLMSCATI (Table 4) and the slt-II promoter / cat fusion pLMSCATII (Table 4) were compared at different iron concentrations in the growth media (Table 8). At low iron concentrations, both strains produced comparable levels of CAT. However,

### Figure 11 Legend

**Shiga-like toxin promoter / cat transcriptional fusions.** The cat gene was placed under the transcriptional control of the stx promoter, which contains a Fur repressor binding site, in the operon fusion pLMSCATI. In pLMSCATII, the cat gene was placed under the transcriptional control of the slt-II promoter. Lastly, the cat gene was placed under the transcriptional control of the lac promoter in the operon fusion pLMSCATC.

## **pLMSCATI**

pKK232-8



## **pLMSCATI<sup>II</sup>**

pKK232-8



## **pLMSCATC**

pKK232-8



**Table 8. Shiga-like toxin promoter transcriptional efficiencies**

Plasmid construct <sup>a</sup>	Promoter	Iron	Amount of CAT enzyme <sup>b</sup> (mean $\pm$ 2 SEM)
pLMSCATI	<u>stx</u>	+	248.5 $\pm$ 119.8 <sup>c</sup>
		-	1201.9 $\pm$ 440.2
pLMSCATII	<u>slt-II</u>	+	702.6 $\pm$ 97.2
		-	893.4 $\pm$ 213.8
pLMSCATC	<u>lac</u>	+	3988.9 $\pm$ 243.0
		-	3027.8 $\pm$ 218.8

<sup>a</sup> As described in Table 4. Four samples tested per group.

<sup>b</sup> CAT enzyme ( $\mu$ g) produced per ml. Mean value  $\pm$  2 standard errors of the mean.

<sup>c</sup> Significantly different ( $p < 0.05$ ) by Student's unpaired t test from value for the same construct grown in the absence of added iron.

when grown in high-iron containing media, the stx promoter fusion produced CAT enzyme levels several-fold less than that of the slt-II promoter fusion. These data confirm the iron-regulated nature of stx promoter activity and establish that the constitutive activity of the slt-II promoter is comparable to the derepressed activity of the stx promoter. Therefore, it is likely that the significant differences observed in the production of SLT-I and SLT-II by toxinogenic clinical isolates of E. coli are not directly related to differences in the transcriptional efficiencies of the stx/slt-I and slt-II/slt-IIv promoters.

**Shiga-like toxin promoter / structural gene chimeras.** To facilitate the subsequent study of the role of iron-regulation of the Shiga-like toxins, two SLT operon chimeras were constructed. The first hybrid, pLMS100 (Figure 12), was comprised of the stx promoter transcriptionally fused to the structural genes for SLT-II. The second, reciprocal hybrid, pLMS200 (Figure 12), was created such that the structural genes for Shiga toxin were placed under the transcriptional control of the slt-II promoter. When grown in low iron concentrations, equivalent levels of SLT-II and Shiga toxin were produced by E. coli DH5 $\alpha$  strains harboring pLMS100 or pLMS200, respectively (Table 9). The production of SLT-II by E. coli DH5 $\alpha$ (pLMS100) was regulated by iron concentrations in the growth media (Table 9). In contrast, Shiga toxin was constitutively produced by E. coli DH5 $\alpha$  (pLMS200) irrespective of the iron level in the media (Table 9). E. coli DH5 $\alpha$

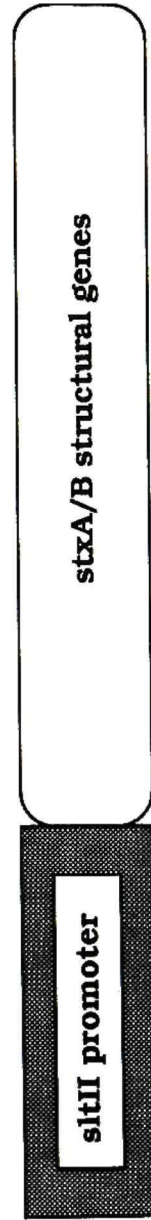
### Figure 12 Legend

**Shiga-like toxin promoter / structural gene chimera constructs.** The structural genes for slt-II were placed under the transcriptional control of the stx promoter, which contains a Fur repressor binding site, in the operon fusion pLMS100. In pLMS200, the structural genes for stx were placed under the transcriptional control of the slt-II promoter.

**pLMS100** Replacement of *slt-II* promoter with *stx* promoter



**pLMS200** Reciprocal exchange



**Table 9. Iron-regulation of Shiga-like toxin gene constructs**

<u>E. coli</u> strain	<u>fur</u>	Plasmid <sup>a</sup>	Iron	Cytotoxicity <sup>b</sup>
DH5 $\alpha$	+	pLMS100	+	10 <sup>4</sup>
DH5 $\alpha$	+	pLMS100	-	10 <sup>5</sup>
DH5 $\alpha$	+	pLMS200	+	10 <sup>5</sup>
DH5 $\alpha$	+	pLMS200	-	10 <sup>5</sup>
H1618	-	pLMS100	-	10 <sup>5</sup>
H1618	-	pNAS13	+	10 <sup>7</sup>
H1618	-	pNAS13	-	10 <sup>7</sup>

<sup>a</sup> As described in Table 4. Four samples tested per group.

<sup>b</sup> Log<sub>10</sub> 50 % cytotoxic dose (CD<sub>50</sub>) per ml of sonically disrupted E. coli strains transformed with the respective plasmid. Cytotoxicity represents CD<sub>50</sub> per ml observed for all four samples tested.

strains containing these promoter / structural gene chimeras were used in latter experiments to examine the in vivo significance of iron-regulation of the Shiga-like toxins.

### **III. Effects of Shiga-like toxin in a murine model of EHEC infection.**

A third major objective of this dissertation project was to assess the in vivo effects of promoter efficiency and iron-regulation of the Shiga-like toxins.

#### **Infection of mice with Shiga-like toxin-producing EHEC O157:H7.**

Recombinant plasmids containing either the Shiga toxin operon or the SLT-II operon were introduced by electroporation into streptomycin-resistant EHEC O157:H7 87-23. This strain lacks genes coding for the Shiga-like toxins (Tarr et al., 1989). Each of the EHEC O157:H7 87-23 transformants colonized the mice. However, those mice orally infected with EHEC O157:H7 87-23(pNN103), which produces SLT-II, died four to five days post-feeding (Table 10). By contrast, mice fed either EHEC O157:H7 87-23(pNAS13), which produces Shiga toxin, or EHEC O157:H7 87-23(pBR329), the vector control, survived the oral infection (Table 10). These data suggest that SLT-II, but not Shiga toxin, can act as a virulence factor to cause death of these animals.

**Table 10 Legend**

- a As described in Table 4. Four samples tested per group.
- b  $\text{Log}_{10}$  50% cytotoxic dose ( $\text{CD}_{50}$ ) per ml of sonically disrupted E. coli strain transformed with the plasmid construct. Cytotoxicity represents  $\text{CD}_{50}$  per ml observed for all four samples tested.
- c Below the limit of detection, less than  $10^1 \text{CD}_{50}$ .

Abbreviation: n/a, not applicable.

Table 10. Effects of Shiga-like toxin-producing *E. coli* (*Stx*<sup>I</sup>) on streptomycin-treated mice

Host strain	<u>fur</u>	Plasmid <sup>a</sup>	Toxin genes	Vector	Cytotoxicity <sup>b</sup>	Death
O157:H7	+	pNN103	<u>slt</u> -IIA/ <u>slt</u> -IIB	pBR328	10 <sup>5</sup>	YES
O157:H7	+	pNAS13	<u>stxA</u> / <u>stxB</u>	pBR329	10 <sup>7</sup>	NO
O157:H7	+	pBR329	none	n/a	BLD <sup>c</sup>	NO
DH5 $\alpha$	+	pNN103	<u>slt</u> -IIA/ <u>slt</u> -IIB	pBR328	10 <sup>4</sup>	NO
DH5 $\alpha$	+	pMJ100	<u>slt</u> -IIA/ <u>slt</u> -IIB	pBS	10 <sup>6</sup>	YES
DH5 $\alpha$	+	pNAS13	<u>stxA</u> / <u>stxB</u>	pBR329	10 <sup>7</sup>	NO
DH5 $\alpha$	+	pBS	none	n/a	BLD <sup>c</sup>	NO
DH5 $\alpha$	+	pBR329	none	n/a	BLD <sup>c</sup>	NO

Table 10 (continued). Effects of Shiga-like toxin-producing *E. coli* (Str<sup>r</sup>) on streptomycin-treated mice

Host strain	<u>fur</u>	Plasmid <sup>a</sup>	Toxin genes	Vector	Cytotoxicity <sup>b</sup>	Death
DH5α	+	pLMS40	<u>slt-IIA</u> / <u>stxB</u>	pBS	10 <sup>4</sup>	NO
DH5α	+	pLMS60	<u>slt-IIvA</u> / <u>stxB</u>	pBS	10 <sup>4</sup>	NO
DH5α	+	pLMS100	<u>stx</u> promoter/ <u>slt-II</u> (AB)	pBR329	10 <sup>5</sup>	YES
DH5α	+	pLMS200	<u>slt-II</u> promoter/ <u>stxB</u> (AB)	pBR329	10 <sup>5</sup>	NO
JE5505	+	pNAS13	<u>stxA</u> / <u>stxB</u>	pBR329	10 <sup>7</sup>	NO
H1618	-	pMJ100	<u>slt-IIA</u> / <u>slt-II</u> B	pBS	10 <sup>6</sup>	YES
H1618	-	pNAS13	<u>stxA</u> / <u>stxB</u>	pBR329	10 <sup>7</sup>	NO

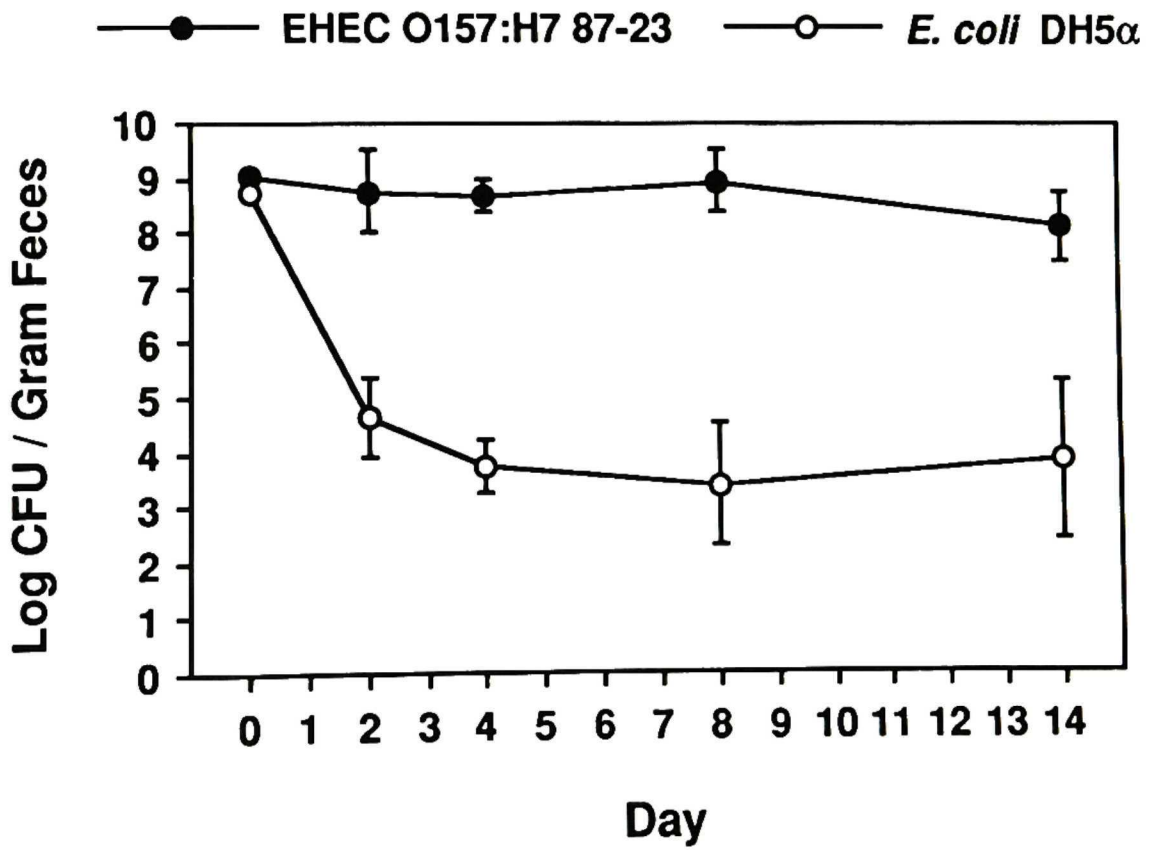
### **Infection of mice with Shiga-like toxin-producing E. coli DH5 $\alpha$ .**

Streptomycin-resistant E. coli DH5 $\alpha$  harboring recombinant plasmids, which contained genes coding for either Shiga toxin or SLT-II, were used in further experiments with orally infected streptomycin-treated mice (Table 10). Those animals fed E. coli DH5 $\alpha$ (pMJ100), which contains genes for SLT-II in a high expression pBS vector, died four to five days post-feeding. No effect was observed in mice fed the control strains, E. coli DH5 $\alpha$ (pBS) or E. coli DH5 $\alpha$ (pBR329). These experiments supported the conclusion that SLT-II is a virulence factor which can cause death of orally infected animals.

When mice were fed E. coli DH5 $\alpha$ (pNN103), which contained the SLT-II operon in a lower expression pBR329 vector, the animals became colonized but did not die. This result indicates that the level of SLT-II production by E. coli DH5 $\alpha$  influences the course of oral infection. Moreover, the background E. coli strain also appeared to affect the outcome of oral infection; EHEC O157:H7 87-23 (pNN103) and E. coli DH5 $\alpha$ (pNN103) produced comparable *in vitro* levels of SLT-II, but only EHEC O157:H7 87-23(pNN103) killed mice. Results of competitive colonization experiments between EHEC O157:H7 87-23 and E. coli DH5 $\alpha$  indicated that EHEC O157:H7 87-23 is a better colonizer of the mouse intestinal tract than E. coli DH5 $\alpha$  (Figure 13). For this, and perhaps other, reasons, a higher production of cytotoxin by E. coli DH5 $\alpha$  appears necessary to mimic the effects on mice observed with EHEC O157:H7 strain 87-23.

### Figure 13 Legend

**Competitive colonization assay.** Streptomycin-treated mice were fed approximately  $10^{10}$  colony forming units (CFU) each of EHEC O157:H7 87-23 and E. coli DH5 $\alpha$  on Day 0. The amount of bacteria persisting in the feces of each strain was recorded over a two week time period. Data points represent the mean  $\log_{10}$  CFU per gram feces for that strain  $\pm$  2 standard errors of the mean (n=9).



Bacteria containing Shiga toxin genes in vector pBR329 did not cause death of infected mice (Table 10). This was a surprising finding, considering that E. coli DH5 $\alpha$ (pNAS13) was 10-fold and 1000-fold more cytotoxic for Vero cells than the SLT-II clones, E. coli DH5 $\alpha$ (pMJ100) and E. coli DH5 $\alpha$ (pNN103), respectively. In addition, it is well established that purified Shiga toxin can kill mice when injected intravenously.

**Effect of toxin release on the virulence of Shiga toxin-producing E. coli.** One possible explanation for the observation that Shiga toxin-producing E. coli DH5 $\alpha$  did not kill mice was that release by E. coli DH5 $\alpha$  of Shiga toxin was minimal compared to SLT-II. Indeed, Shiga toxin/SLT-I is primarily cell-associated, whereas SLT-II is found predominantly in culture supernatants. To examine this possibility, the Shiga toxin plasmid pNAS13 was transformed into E. coli JE5505, a mutant K-12 strain which expresses a periplasmic leaky phenotype. The cytotoxic activity of culture supernatants of E. coli JE5505(pNAS13) was 10 to 100-fold higher than that of E. coli DH5 $\alpha$ (pNAS13), a finding which indicates that Shiga toxin is released in greater relative quantities *in vitro* by the E. coli JE5505 background than the E. coli DH5 $\alpha$  background. In spite of this increased release of Shiga toxin, E. coli JE5505(pNAS13) did not kill the streptomycin-treated mice (Table 10).

**Effect of iron on the virulence of Shiga toxin-producing *E. coli*.** A second possible explanation for the observation that Shiga toxin-producing *E. coli* do not kill mice is that iron represses toxin production *in vivo*. It should be noted that this explanation seems unlikely because the level of available iron in the mouse intestine is believed to be extremely low, and such an environment would increase rather than depress Shiga toxin production. Nevertheless, to eliminate the possibility that *E. coli* DH5 $\alpha$ (pNAS13) is incapable of killing these mice because of the iron-regulated nature of Shiga toxin, the genes for Shiga toxin were introduced into *E. coli* H1618, a K-12 mutant strain which possesses a Fur null phenotype. Cytotoxicity assays confirmed that Shiga toxin was constitutively produced *in vitro* by *E. coli* H1618 irrespective of iron levels in the growth media (Table 9). Nonetheless, mice fed *E. coli* H1618(pNAS13) survived (Table 10) which suggests that iron-regulation of Shiga toxin production is not responsible for the avirulence of Shiga toxin-producing *E. coli*.

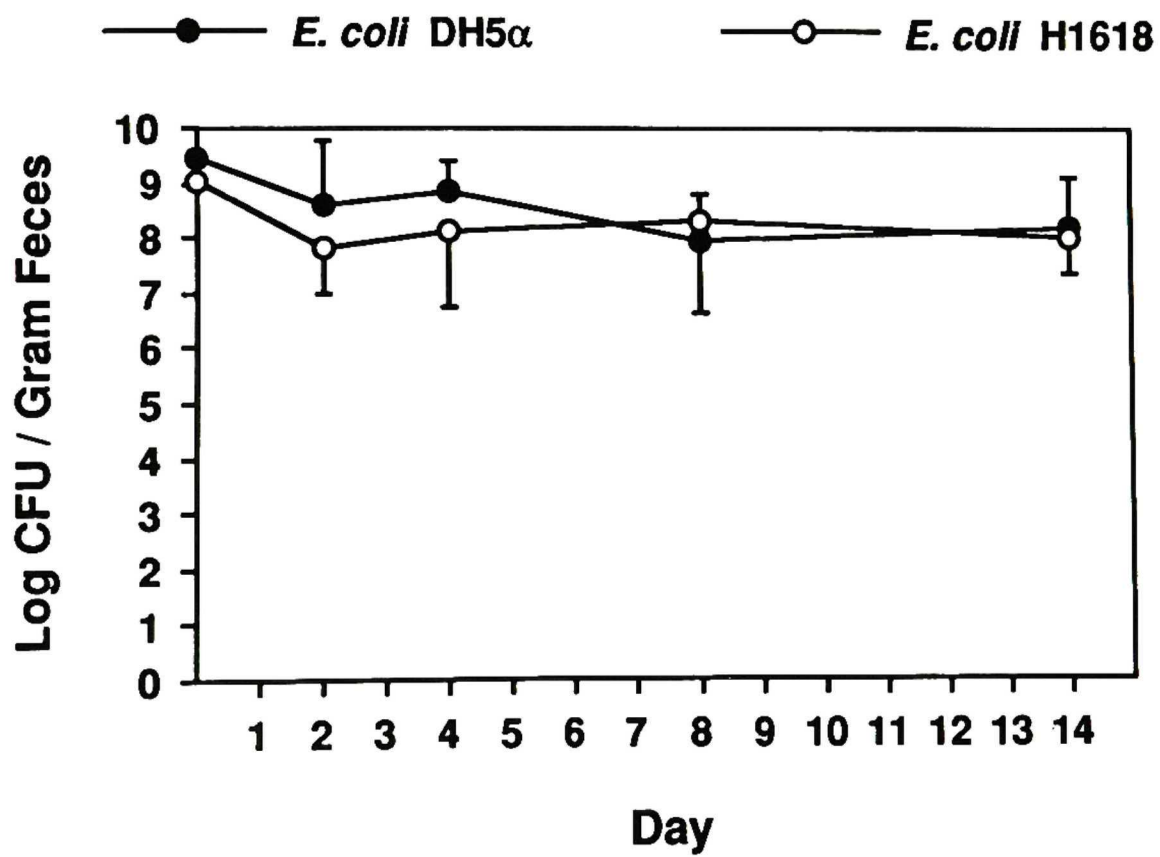
Another way of testing the relevance *in vivo* of iron-regulation was to evaluate the virulence of *E. coli* DH5 $\alpha$  producing SLT-II in an iron-regulated manner. This was accomplished by feeding streptomycin-treated mice *E. coli* DH5 $\alpha$  containing the *stx* promoter / *slt-II* structural gene chimera pLMS100. As noted earlier, when this construct was transformed into the *fur*<sup>+</sup> host *E. coli* DH5 $\alpha$ , SLT-II production was iron-regulated (Table 9). When pLMS100 was expressed by the *fur*<sup>-</sup> host *E. coli* H1618, SLT-II production was, like wild-type, constitutively

produced irrespective of the iron concentration of the growth media (Table 9). Streptomycin-treated mice died four to five days post-feeding when infected with either *E. coli* DH5 $\alpha$ (pLMS100) (Table 10) or *E. coli* H1618(pLMS100) (Table 9). It should be noted that competitive colonization experiments between *E. coli* DH5 $\alpha$  and *E. coli* H1618 revealed that these strains were equally efficient colonizers of the mouse intestinal tract (Figure 14). Thus, SLT-II is capable of killing mice whether or not its production is iron-regulated.

**Infection of mice with *E. coli* DH5 $\alpha$  containing Shiga-like toxin subunit chimeras.** Operon fusions in which Shiga-like toxin A subunit genes were transcriptionally fused to heterologous SLT B subunit genes have been previously described. In this study, *E. coli* DH5 $\alpha$  containing two such fusions in the high expression pBS vector, pLMS40 and pLMS60 (Table 4), were fed to streptomycin-treated mice (Table 10). No detrimental effects were seen in mice fed *E. coli* DH5 $\alpha$  which harbored genes for either the SLT-IIA / Shiga toxin B subunit hybrid or the SLT-IIvA / Shiga toxin B subunit hybrid. These data suggest that the reason Shiga toxin-producing *E. coli* fail to kill mice relates to the B subunit of Shiga toxin. This hypothesis could not be definitively proven because no functional Shiga toxin A / SLT-IIB or Shiga toxin A / SLT-IIvB subunit chimeras were available.

### Figure 14 Legend

**Competitive colonization assay.** Streptomycin-treated mice were fed approximately  $10^{10}$  colony forming units (CFU) each of E. coli DH5 $\alpha$  and E. coli H1618 on Day 0. The amount of bacteria persisting in the feces of each strain was recorded over a two week time period. Data points represent the mean  $\log_{10}$  CFU per gram feces for that strain  $\pm$  2 standard errors of the mean (n=9).



## DISCUSSION

### I. **Transcriptional regulation studies.**

The first specific aim of this dissertation project was to characterize the transcriptional organization of the SLT-II operon. A number of reports in recent years have provided information on the genetics of the cytotoxins comprising the Shiga toxin family. The nucleotide sequences of the structural genes for Shiga toxin, SLT-I and SLT-II had been determined prior to the research conducted here. Primer extension and S1 nuclease protection analyses were used to identify the slt-II promoter, and computer nucleotide homology search was used to identify the slt-II transcription terminator. These structures further defined the organization of the SLT-II operon proposed from nucleotide sequence analysis and permitted a comparison to the promoter and transcription terminator identified by other investigators for the SLT-I operon. The Shiga-like toxin operons were examined to determine if differences existed at the transcriptional level which might account for the differences between the production of Shiga toxin/SLT-I and SLT-II with respect to level and response to iron concentrations in the growth environment.

While the promoter mapping experiments were in progress, nucleotide sequence data of SLT-IIv became available which revealed that the regions upstream of the SLT-II and SLT-IIv structural genes were 85% homologous. This observation suggested that the promoters for these operons might be similar.

Indeed, the putative slt-IIv promoter, also identified by primer extension analysis, was demonstrated to be virtually identical to the putative slt-II promoter.

The slt-II/slt-IIv promoter had less nucleotide sequence homology than the stx/slt-I promoter to a consensus sequence established from a compilation of defined E. coli promoters. Those promoters lacking homology with this consensus sequence are generally less efficient at initiating transcription than promoters with a high degree of homology. In turn, those genes transcribed from less efficient, or "weaker", promoters are usually expressed at lower relative levels. Nucleotide sequence comparison predicted that the slt-II/slt-IIv promoter was weaker than the stx/slt-I promoter and therefore, the SLT-II or SLT-IIv operons when compared to the Shiga toxin/SLT-I operon should be expressed at lower levels.

As noted earlier, the production of Shiga toxin/SLT-I occurs at high levels and is influenced by levels of iron in the growth media. In contrast, SLT-II and SLT-IIv are produced at moderate levels, and this production is not iron-regulated. These observations taken in conjunction with the predictions from the promoter nucleotide sequence comparison led to the initial hypothesis that the promoters for these toxin operons dictated both the capacity for iron-regulation and the level of cytotoxin production.

While the studies described here were in progress, Calderwood et al. reported that the iron-regulation of the SLT-I was, in fact, a result of the binding of

a Fur protein / iron co-repressor complex to a site within the slt-I promoter which prevents transcription of the toxin genes (Calderwood et al., 1987). In this investigation, experiments using E. coli fur mutant strains definitively demonstrated that the Fur protein plays no role in the regulation of SLT-II operon expression. This result was consistent with the fact that no Fur binding site could be identified within the slt-II/slt-IIv promoter.

The negative transcriptional regulation of both siderophore-based iron uptake systems and SLT-I production in E. coli by the Fur protein is an example of the coordinate expression of genes in response to environmental signals that pathogenic organisms may use to recognize entry into the mammalian host. The reason why only certain members of the Shiga toxin family are iron-regulated is unclear.

Precedence exists for the interaction of positive regulatory factors with weak promoters to enhance the transcription of the genes from these inefficient promoters. To determine if one such regulatory protein, the cyclic AMP regulatory protein (CRP), might be involved in the regulation of transcription of the SLT-II operon, SLT-II production was assessed in an E. coli crp mutant strain deficient in CRP. The cyclic AMP regulatory protein did not affect SLT-II production which further suggests that the transcription of the SLT-II operon is not regulated by protein factors.

Previous reports on the mechanism of transcription of the SLT-I operon provided some suggestive evidence that a second promoter might exist within the downstream sequences of the slt-IA open reading frame to initiate the independent transcription of the B subunit gene. Furthermore, Northern blot analysis of the transcription of the Shiga toxin operon by Kozlov *et al.* revealed both a polycistronic message and a B subunit message. An independent transcriptional start site for stx/slt-IB could lead to increased expression of the B subunit gene, which would account for greater amounts of B subunit protein relative to that of the A subunit. This differential subunit production could help explain the single A subunit to multiple B subunit protein composition observed in the holotoxin. However, the results of Northern blot and primer extension analyses of slt-II reported here clearly indicated that transcription of the SLT-II operon occurs as a single unit. Thus, either the Shiga toxin/SLT-I operon is transcribed differently than the SLT-II operon, or a discrepancy in the Northern blot data generated here and in Kozlov's laboratory exists.

There are several possible regulatory mechanisms to account for the subunit stoichiometry of the holotoxin. The regulation of protein subunit synthesis in the SLT-II operon may be analogous to that for the cholera toxin operon. Cholera toxin, which exists as a single A subunit with five B subunits, is also translated from a polycistronic message. Thus, explanations proposed for the translational control of the cholera toxin operon may be applicable to the SLT-II

operon. The translation of the SLT-II B subunit open reading frame from the polycistronic mRNA could occur more efficiently through the action of an independent putative ribosome binding site for slt-IIB located in the intercistronic gap. Alternatively, the conformation assumed by the slt-II RNA transcripts in the region coding for the A subunit could be different from that for the B subunit. A difference in the secondary structure of mRNA between these regions could result in the differential synthesis of proteins from a polycistronic message.

The working hypothesis of this laboratory is that the single A to multiple B subunit stoichiometry of the SLT holotoxin occurs because more B subunits than A subunits are translated (as reviewed in O'Brien and Holmes, 1987). However, another possibility is that the A subunit and the B subunit are produced in equivalent amounts. If so, holotoxin assembly would leave an excess of A subunit in an unassociated state. Alternatively, post-translational modifications might occur which render the B subunit less susceptible than the A subunit to protease degradation. These hypotheses could be addressed using monospecific antibodies directed against either the A subunit or the B subunit to determine the relative quantities of subunit protein production.

## **II. Comparison of Shiga-like toxin promoter efficiencies.**

The second specific aim of this dissertation project was to compare the transcriptional efficiencies of the stx/slt-I and slt-II/slt-IIv promoters. Although these

promoters were quite different in nucleotide sequence, whether these sequence differences dictated functionally dissimilar promoters remained to be determined. The isolation of these promoter sequences was integral to examining Shiga-like toxin promoter function. Unfortunately, no naturally occurring restriction endonuclease sites existed that would enable a precise excision of the promoters. Therefore, sequences flanking the promoters were targeted for oligonucleotide-directed, site-specific mutagenesis to create convenient restriction endonuclease sites. The creation of these convenient restriction sites permitted the construction of SLT promoter-deletion subclones. Analysis of these subclones by cytotoxicity assay confirmed that the putative sequences mapped by primer extension and S1 nuclease protection studies were indeed the functional promoters for the SLT-II and SLT-IIv operons.

To test the hypothesis that differences in the level of cytotoxin production of Shiga toxin/SLT-I and SLT-II reflect a difference in the transcriptional efficiency of the promoters, the stx and slt-II promoters were examined. Surprisingly, results of the promoter / cat fusion studies indicated that the transcriptional activity of the slt-II promoter is equivalent to that of the stx promoter under low iron growth conditions. Therefore, the significantly higher in vitro cytotoxic activity of Shiga toxin/SLT-I as compared to SLT-II and SLT-IIv is not directly related to differences in the transcriptional efficiencies of the stx/slt-I and slt-II/slt-IIv promoters.

There are yet several possible mechanisms to account for the difference in the level of toxin production between the Shiga-like toxins. First, the steady-state amounts of RNA transcripts and protein products of Shiga toxin/SLT-I and SLT-II might be different as a result of regulation at the translational or post-translational level. Second, there might be differences between the Shiga-like toxins in the efficiency of holotoxin assembly. If, in contrast to that of Shiga toxin/SLT-I, the holotoxin assembly of SLT-II were to leave a large excess of unassociated SLT-II B subunits, a competitive inhibition of holotoxin binding to the target receptor by free B subunits might occur. This would result in a lower level of cytotoxic activity of SLT-II compared to Shiga toxin/SLT-I. Third, Shiga toxin/SLT-I and SLT-II might have different subunit protein stoichiometries. Fourth, the lower level of SLT-II could be a result of reduced affinity for the globotriosyl receptor compared to Shiga toxin/SLT-I. Fifth, the efficiency of translocation of the enzymatically active A subunit across the cytoplasmic membrane could differ between Shiga toxin/SLT-I and SLT-II. This would also result in a lower level of cytotoxic activity of SLT-II compared to Shiga toxin/SLT-I.

### **III. In vivo analysis of Shiga-like toxins.**

The third specific objective of this dissertation project was to determine the in vivo significance of the differences in the transcriptional regulation of Shiga toxin/SLT-I and SLT-II with respect to promoter efficiency and iron-regulation. A

streptomycin-treated mouse model was being used in our laboratory to examine bacterial colonization by an EHEC strain. Oral infection of these animals by bacteria expressing plasmid-encoded Shiga-like toxin genes was a practical system for in vivo toxin delivery and an ideal method of examining the in vivo effects of SLT operon chimeras created in the regulation studies.

A clinical isolate from an outbreak of hemorrhagic colitis, EHEC O157:H7 87-23, was selected as the bacterial host. EHEC O157:H7 87-23 is to date the only disease-associated EHEC strain isolated that, for reasons unknown, does not contain, or has lost, Shiga-like toxin genes as determined by DNA hybridization (Tarr et al., 1989). Therefore, this isolate represents a unique vehicle for assessing the role of the SLTs in the virulence of EHEC. Unfortunately, just as the initial experiments using EHEC O157:H7 87-23 were completed, it was recognized that the introduction of recombinant plasmids into a wild-type E. coli strain was not prescribed under the recombinant DNA guidelines established by the National Institutes of Health in the Federal Register. Since other investigators had shown that even laboratory E. coli K-12 strains can colonize a streptomycin-treated mouse, the examination of Shiga-like toxin expression from recombinant DNA constructs was continued in the K-12 strain, E. coli DH5 $\alpha$ .

Oral infections of mice with either EHEC O157:H7 87-23 or E. coli DH5 $\alpha$  revealed that SLT-II, but not Shiga toxin, could function as a virulence determinant. The results of infection with SLT-II-producing E. coli DH5 $\alpha$  further supported the

conclusion that production of SLT-II alone was sufficient to cause death in mice.

That Shiga toxin-producing bacteria were unable to kill mice was surprising for two reasons: Shiga toxin-producing strains were 10-fold to 1000-fold more cytotoxic for Vero cells than the SLT-II clones, and it has been demonstrated that purified Shiga toxin intravenously injected is capable of killing mice. Several hypotheses were proposed to account for the observation that Shiga toxin-producing EHEC O157:H7 87-23 or Shiga toxin-producing *E. coli* DH5 $\alpha$  were unable to cause death of orally infected streptomycin-treated mice. First, it was conceivable that an even higher level of Shiga toxin production by *E. coli* DH5 $\alpha$  was required to kill these animals. Attempts to clone Shiga toxin genes into pBS have, however, been unsuccessful. Perhaps expression of Shiga toxin at such high levels is detrimental to the bacterial host. Second, the concern was addressed that the cytotoxic activity on Vero cells of whole cell lysates of Shiga toxin-producing bacteria might not reflect the actual amount of toxin elaborated by these strains *in vivo*. Results of feeding experiments using a periplasmic leaky strain that produced Shiga toxin suggested that the efficiency of *in vivo* cytotoxin release did not influence the outcome of infection. Third, the possibility was tested that Shiga toxin-producing *E. coli* failed to kill mice because iron represses toxin production *in vivo*. This prospect was eliminated on the basis of data obtained when mice were fed a *fur*<sup>-</sup> *E. coli* strain expressing the genes for Shiga toxin and on the basis of studies with the *stx* promoter / *slt*-II structural gene

chimera pLMS100. Results from both types of experiments indicated that iron-regulation of toxin production *in vitro* did not influence the course of *in vivo* infection. Specifically, neither the deregulation of iron repression of Shiga toxin nor the regulation of SLT-II production by iron altered the course of infection. Fourth, although the B subunits of Shiga toxin and SLT-II have been shown to recognize the same eucaryotic receptor, globotriosyl ceramide, it is possible that the Shiga toxin B subunit exhibits much less affinity for the receptor *in vivo* than does SLT-II. Results of the infection of mice with *E. coli* DH5 $\alpha$  containing Shiga-like toxin chimeras suggested but did not prove that the reason these bacteria are not capable of killing mice relates to the B subunit of Shiga toxin.

It therefore remains unclear why strains that produce SLT-II, but not Shiga toxin, are able to cause death in mice in this animal model. Nonetheless, these results are consistent with epidemiological data which indicate that EHEC O157:H7 strains containing genes for SLT-II alone are more likely to cause a diarrheal syndrome progressing to HUS or TTP than strains which contained genes for both SLT-II and SLT-I or SLT-I alone. What remains perplexing is why an EHEC strain that produces both SLT-I and SLT-II should be less virulent than a strain that produces only SLT-II. This apparent attenuation in virulence of SLT-I-producing EHEC compared to SLT-II-producing EHEC might be explained by the demonstration that commercially available human immune globulin preparations contain neutralizing antibodies to Shiga toxin and SLT-I, but not SLT-II (Ashkenazi,

et al., 1988). In addition, it has been proposed that the production of SLT-II may represent a phenotype closely linked to an as yet undetermined virulence factor. This hypothesis seems unlikely because in this study E. coli strains transformed with plasmids containing the SLT-II operon and little flanking DNA from the EHEC strain were virulent when fed to mice.

#### IV. Summary.

In conclusion, the promoters for the SLT-II and SLT-IIv operons were identified and found to be identical. The transcriptional efficiency of the slt-II/slt-IIv promoter was shown to be equivalent to the stx/slt-I promoter under low iron growth conditions. However, the activity of the slt-II/slt-IIv promoter, in contrast to that of the stx/slt-I promoter, was not regulated by the Fur protein and its iron co-repressor. The SLT-II operon was demonstrated to be transcribed as a single polycistronic message, and no slt-IIB independent transcript was demonstrated. These data indicate that the difference in the level of production between Shiga toxin/SLT and SLT-II as well as the single A subunit to multiple B subunit stoichiometry of the SLT-II holotoxin are not consequences of regulation at the transcriptional level.

In contrast to Shiga toxin-producing bacteria, SLT-II-producing bacteria were capable of causing death of orally infected streptomycin-treated mice. There was suggestive evidence that Shiga toxin-producing bacteria are unable to kill these mice because of the nature of the Shiga toxin B subunit. No role for iron-regulation of Shiga toxin/SLT-I transcription in EHEC or S. dysenteriae 1 pathogenesis was found.

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