Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family

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Escherichia coli strain H.I.8 (O128:B12) produces low levels of a Shiga-like toxin (SLT) which we have called SLTIIva because of its close relationship with SLTIIv. The Vero cell cytotoxicity of SLTIIva is neutralized by antisera against SLTII and SLTIIv but not by antisera against SLTI. These data indicate that the SLT of strain H.I.8 is a member of the SLTII family. Since SLTIIva shares with SLTIIv the property of having low cytotoxicity to HeLa cells compared with Vero cells, it is appropriate to consider both toxins as variants of SLTII. SLTIIva differs from SLTIIv in that it is more heat-stable. Further, SLTIIv-producing strains of *E. coli* have only been isolated from pigs while the SLTIIva-producing *E. coli* strain examined in this study was isolated from a human infant with diarrhoea. The genes for this SLT were cloned from a cosmid library of total cellular DNA by screening recombinants for Vero cell toxicity and with a DNA probe derived from SLTIIv structural genes. Nucleotide sequence analysis was performed on a 2.0 kb *Ava*II–*Hinc*II fragment which encodes the toxin gene. The nucleotide sequence data confirm the close relationship between SLTIIva and SLTIIv: they have 98% nucleotide sequence homology in the B subunit gene and 70.6% homology in the A subunit gene. Comparison of DNA sequences indicated that SLTIIva was most closely related to SLTIIv, closely related to SLTII and less closely related to SLTI.

Introduction

Strains of *Escherichia coli* which produce Shiga-like toxins (SLTs) or Vero toxins (VTs) are associated with infant diarrhoea, haemorrhagic colitis and the haemolytic uraemic syndrome in man, dysentery in calves and oedema disease of pigs (Konowalchuk *et al.*, 1977; O'Brien *et al.*, 1982; Smith *et al.*, 1983; Karmali *et al.*, 1985; O'Brien & Holmes, 1987; Smith & Scotland, 1988). All SLTs have delayed Vero cell toxicity and share a common mechanism of action with Shiga toxin of *Shigella dysenteriae* type 1.

SLTs which have been well characterized include SLTI (O'Brien *et al.*, 1982), SLTII (Strockbine *et al.*, 1986; Jackson *et al.*, 1987a), and a variant of SLTII (SLTIIv) which is produced by *E. coli* strains associated

with oedema disease (Marques *et al.*, 1987; Weinstein *et al.*, 1988; Gyles *et al.*, 1988). SLTI is identical to Shiga toxin of *Shigella dysenteriae* type 1 in immunological cross-reactivity and biological activity and is nearly identical to this toxin in nucleotide and amino acid sequence (Jackson *et al.*, 1987b; Calderwood *et al.*, 1987; De Grandis *et al.*, 1987; Strockbine *et al.*, 1988; Kozlov *et al.*, 1988). SLTII and SLTIIv cross-react immunologically with each other and share 91% nucleotide sequence homology (Marques *et al.*, 1987; Weinstein *et al.*, 1988; Gyles *et al.*, 1988). However, SLTII and SLTIIv are distinct from SLTI and Shiga toxin. SLTII and SLTIIv are not neutralized by polyclonal or monoclonal antisera generated against SLTI and Shiga toxin (Marques *et al.*, 1987; Perera *et al.*, 1988) and only share approximately 55–60% overall nucleotide sequence homology with the latter toxins (Newland *et al.*, 1987; Weinstein *et al.*, 1988; Gyles *et al.*, 1988).

Other SLT or VT variants have been identified from *E. coli* O serogroups 91, 128, and 157 based on differential cytotoxicity to Vero and HeLa cells (Karch &

Abbreviations: SLT, Shiga-like toxin; VT, Vero toxin.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M29153.

Meyers, 1989; Oku *et al.*, 1989; Gannon & Gyles, 1990), reactions with conventional (Smith *et al.*, 1987) and synthetic oligonucleotide probes (Karch & Meyers, 1989; Brown *et al.*, 1989), neutralization of toxicity with SLT-specific antisera (Dickie *et al.*, 1989; Gannon & Gyles, 1990) and analysis of antigenic components by the Ouchterlony double gel diffusion assay (Oku *et al.*, 1989). Further, recent evidence (Dickie *et al.*, 1989) suggests that VT2, first described by Scotland *et al.* (1985), is not identical to SLTII, described by Strockbine *et al.* (1986). In this study we report the cloning and nucleotide sequence analysis of an SLTII variant, which is different from the variant described previously (Marques *et al.*, 1987; Weinstein *et al.*, 1988; Gyles *et al.*, 1988). The new toxin is designated SLTIIva, indicating that it is closely related to SLTIIv.

Methods

Bacterial strains and plasmids. SLT-producing *E. coli* strains examined are shown in Table 1. Plasmids and bacteriophages employed in the construction of the cosmid library, cloning, subcloning and DNA sequencing are shown in Table 2, as are the recombinant plasmids used in the study. *E. coli* K12 strains DH5 α (from BRL) and XL1-Blue (from Stratagene) were used as hosts in cloning and subcloning experiments. The *E. coli* K12 strain C600 λ^- , which originated from the late H. W. Smith (Houghton Poultry Research Station, Houghton, Cambridgeshire, UK), was used in the propagation of bacteriophages.

Media, enzymes, biochemicals and radionuclides. Luria broth (LB), or brain heart infusion broth or agar, were used for routine culturing of bacteria. Media were supplemented with X-Gal, IPTG and ampicillin

for growth or selection of recombinants, as required. Restriction endonucleases, exonuclease III, S1 nuclease, T4 ligase, calf intestinal phosphatase, the Klenow enzyme and T4 kinase were purchased from BRL and T7 polymerase from Pharmacia. Radionuclides were purchased from Amersham.

Cloning, subcloning and restriction mapping. For small-scale preparation of plasmid DNA the alkaline lysis method of Birnboim & Doly (1979) was used with modifications described by Morelle (1989); for large-scale plasmid preparation, either the Triton lysis method (Ausubel *et al.*, 1987) or the rapid boiling procedure of Holmes & Quigley (1981) was employed. In some experiments plasmid DNA was further purified by centrifugation in caesium chloride gradients. Total cellular DNA was isolated by the method of Ausubel *et al.* (1987) and procedures for cosmid cloning and *in vitro* packaging of ligated DNA were those of Maniatis *et al.* (1982). Methods described by Ausubel *et al.* (1987) were employed in the construction of the restriction map of insert DNA in pVGT10 (Fig. 1).

DNA hybridization studies. Plasmid DNA was transferred from agarose gels to nylon membranes (Hybond-N, Amersham) by a vacuum unit (VacuGene, Pharmacia-LKB) and total DNA to nylon membranes with a slot-blot apparatus (BioRad). Restriction fragments cloned into pUC19 or recovered from gel slices by electro-elution were denatured, mixed with random nucleotide hexamers and labelled with an equal mixture of [α - 32 P]dATP and [α - 32 P]dCTP (3000 Ci mmol $^{-1}$; 110 TBq mmol $^{-1}$) using Klenow enzyme. Synthetic oligonucleotides were end-labelled with [γ - 32 P]dATP (5000 Ci mmol $^{-1}$; 185 TBq mmol $^{-1}$) by T4 kinase. Highly stringent hybridization conditions were employed in all studies. For labelled DNA fragments the prehybridization and hybridization solutions consisted of 50% (v/v) formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 10% (w/v) dextran sulphate, 0.1% sodium dodecyl sulphate (SDS), with denatured salmon sperm at a concentration of 100 μ g ml $^{-1}$ [1 $\times$ Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone; 1 $\times$ SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate]. Membranes were preincubated in this solution for 2 h at 42 $^{\circ}$ C, labelled probe was added and membranes were incubated overnight at 42 $^{\circ}$ C. After hybridization,

Table 1. SLT-producing *E. coli* strains

Strain	Disease	Serotype	SLT	Source or reference
H19	Infant diarrhoea	O26:K60:H11	SLTI	Konowalchuk <i>et al.</i> (1977)
C600(933W)*	None		SLTII	Strockbine <i>et al.</i> (1986)
E32511	Haemolytic uraemic syndrome	O157:H-	'VT2'	Scotland <i>et al.</i> (1985)
412	Oedema disease	O139:K82	SLTIIv	Gyles <i>et al.</i> (1988)
H.I.8	Infant diarrhoea	O128:B12	SLTIIva	Konowalchuk <i>et al.</i> (1977)
B2F1/3	Haemolytic uraemic syndrome	O91:H21	'VT2'	Karmali <i>et al.</i> (1985)

* An *E. coli* K12 C600 strain lysogenic for an SLT-converting bacteriophage from the *E. coli* O157:H7 strain 933.

Table 2. Plasmids and bacteriophages

	Description	Source and/or reference
pHC79	Cosmid cloning vector	BRL; Hohn & Collins (1980)
pUC18 and pUC19	Cloning and sequencing vectors	BRL; Vieira & Messing (1982)
M13 mp18 and mp19	Sequencing vectors	BRL; Yanisch-Perron <i>et al.</i> (1985)
pCG6	pUC18 with a 2.4 kb <i>EcoRI</i> - <i>Bgl</i> II insert; <i>sltIIv</i> ⁺	Gyles <i>et al.</i> (1988)
pVGT5	pHC79 with a 35 kb insert; <i>sltIIva</i> ⁺	This study
pVGT10	pUC19 with a 4.5 kb <i>Hind</i> III insert; <i>sltIIva</i> ⁺	This study
pVGT15	pUC19 with a 2.0 kb <i>Ava</i> II- <i>Hinc</i> II insert; <i>sltIIva</i> ⁺	This study

membranes were given three 10 min washes with a solution of $2 \times \text{SSC}/0.1\%$ SDS at 42°C and two 15 min washes with a solution of $0.1 \times \text{SSC}/0.1\%$ SDS at 65°C . Membranes for hybridization with oligonucleotide probes were prehybridized in $6 \times \text{SSC}$, $5 \times$ Denhardt's solution, 0.05% sodium pyrophosphate, 0.5% SDS, with denatured salmon sperm at a concentration of $100 \mu\text{g ml}^{-1}$, for 1 h at 37°C . Membranes were removed from the solution and placed in hybridization solution ($6 \times \text{SSC}/1\%$ SDS) together with the labelled oligonucleotide probe and incubated overnight at 42°C . After hybridization, membranes were washed three times in $6 \times \text{SSC}/1\%$ SDS for 10 min each wash and once for 5 min with $1 \times \text{SSC}/1\%$ SDS at 42°C . After washing, membranes were dried, covered with plastic wrap and exposed to X-ray film at -70°C .

Biological assays. Assays for cytotoxicity of bacterial culture supernatants in Vero (ATCC CCL 81) and HeLa 229 (ATCC CCL 2.1) cells, tests for neutralization of toxic activity with polyclonal porcine antisera and heat lability tests of toxins were performed as described by Gannon & Gyles (1990). Polyclonal porcine antiserum to SLTIIv was obtained from D. L. MacLeod (Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario).

Bacteriophage induction. Induction of bacteriophages was accomplished by: (1) exposure of cultures to ultraviolet light as described by O'Brien *et al.* (1984); and (2) addition of mitomycin C (Sigma) to mid-exponential-phase cultures (OD_{600} 0.5) to a final concentration of $1 \mu\text{g ml}^{-1}$ (Karch & Bitzman, 1988).

Nucleotide sequence analysis. Restriction fragments of the 4.5 kb *HindIII*–*HindIII* insert (containing the *slt* gene) in recombinant plasmid pVGT10 (see Fig. 1), were subcloned into M13 mp18 and mp19 replicative-form vectors and into pUC19 and pUC18 plasmids for single- and double-stranded DNA sequencing, respectively. For large DNA fragments where no convenient restriction sites could be located, deletions of DNA were created by exonuclease III digestion (Henikoff, 1984). Either a 17-base M13 universal primer or synthetic 17-mer oligonucleotides complementary to the insert DNA (Regional DNA Synthesis Laboratory, Calgary, Alberta, Canada) were used as primers for the dideoxy chain-termination method of sequence analysis (Sanger *et al.*, 1977). T7 DNA polymerase was substituted for the Klenow enzyme in sequencing reactions. The DNA sequence was determined for both strands. For areas where GC compression was noted, 7-deaza-dGTP (US Biochemicals) was used in place of dGTP in chain-termination reactions. The MicroGenie computer program, version 6.0 (Beckman Instruments) was used to analyse and compare the *sltIIva* sequence data with those of other *slt* genes and the predicted amino acid sequence of the SLTIIv A subunit with that of the ricin A chain. Homology comparisons among SLT genes were made using the alignment function of the MicroGenie program. Nucleotide or predicted amino acid sequences of SLT A and B subunits, extending from the proposed signal peptide cleavage site or the N-terminus of the mature polypeptide to the termination codon or C-terminus of the polypeptide, were compared. In the promoter region comparisons are based on optimal sequence alignment of nucleotides 5' to the initiation codon of the A subunit sequence. The nucleotide sequence 3' to the termination codon of the B subunit was compared in the same manner. In certain cases gaps were introduced into nucleotide or amino acid sequences by the computer program to achieve optimal alignment.

Results

Cloning of the *sltIIva* genes

VT assays on the broth culture supernatants from 600 recombinants obtained from the cosmid library of *E. coli*

strain H.I.8 yielded two positive clones. A 1.065 kb fragment of the *sltIIv* gene (from pCG6), which extends from the *EcoRV* site in the A subunit gene to the *PstI* site in the B subunit gene (Weinstein *et al.*, 1988; Gyles *et al.*, 1988), was used as a DNA probe. This probe hybridized with a 4.5 kb fragment from *HindIII*-digested cosmid DNA of the VT⁺ clones. The 4.5 kb *HindIII* fragment of one of the VT⁺ cosmid clones, pVGT5, was subcloned into pUC19 to yield the recombinant plasmid pVGT10. A restriction map of pVGT10 insert DNA is presented in Fig. 1.

To determine the extent of the *sltIIva* genes, various restriction fragments of insert DNA from pVGT10 were subcloned into pUC19 and used to transform *E. coli* strain DH5 α , and the culture supernatants of these transformants were tested in the VT assay. A 2.0 kb region of pVGT10 insert DNA bounded by *AvaII* and *HincII* sites, respectively, was found necessary for VT activity. This DNA fragment was subcloned into pUC19, as described in Fig. 1, to yield pVGT15.

Characteristics of VT produced by recombinant *E. coli* strains

VT in the culture supernatants of the cosmid clone DH5 α (pVGT5) and in DH5 α (pVGT10) and DH5 α (pVGT15) was identical to that of strain H.I.8, in that: (1) it was partially neutralized by polyclonal porcine antisera to SLTIIv and SLTII, but was not neutralized by anti-SLT I (Table 3); (2) VT activity was retained after heating at 80°C for 30 min; and (3) titres in cytotoxicity assays were similar to SLTIIv preparations from *E. coli* 412, in that they were approximately 125-fold greater in Vero than in HeLa 229 cells. Titres in cytotoxicity assays for broth culture supernatants from *E. coli* strains H19(SLT I) and C600(933W)(SLT II) in contrast, were approximately equal in these two cell lines.

Location of *sltIIva* genes in *E. coli* strain H.I.8

Repeated attempts to induce bacteriophages from broth culture supernatants of *E. coli* strain H.I.8 by exposure of cultures to ultraviolet light, or by culture in the presence of mitomycin C, were unsuccessful. In contrast, bacteriophages were readily induced from *E. coli* strains H19 and C600(933W), and C600 lysogens obtained from plaques for these two strains were VT⁺.

To determine the cellular location of *sltIIva* genes, a 770 bp *PstI*–*BglII* fragment internal to the cloned *sltIIva* structural genes (see Fig. 1) was obtained from pVGT15 and used as a probe in DNA hybridization studies with plasmid and total cellular DNA of *E. coli* strain H.I.8.

Fig. 1. Restriction enzyme map of a DNA fragment encoding SLTIIva. A 4.5 kb *HindIII*–*HindIII* fragment obtained from a VT⁺ cosmid clone of *E. coli* strain H.I. 8, pVGT5, was subcloned into pUC19 to give the recombinant plasmid pVGT10. VT activity was determined for *E. coli* DH5 α transformants of recombinant plasmids which contained various restriction fragments of pVGT10. Based on this analysis a 2.0 kb *AvaII*–*HincII* region of DNA necessary for VT activity was identified. This region was subcloned as follows. (1) The left 3.1 kb *HindIII*–*PstI* and adjacent 200 bp *PstI*–*HincII* fragments were sequentially subcloned into pUC19 to reconstitute the left 3.3 kb *HindIII*–*HincII* region (in this construct, pVGT12, insert DNA was bounded by a *HindIII* site and a *HincII*/*SmaI* junction), (2) pVGT12 was then digested with *AvaII* (since the insert contains one and pUC19 contains two *AvaII* sites, three DNA fragments were generated). (3) Fragments were made blunt-ended by S1 nuclease digestion and filling with the Klenow enzyme. (4) The DNA was then digested with *SstI*, which freed the 2.0 kb fragment of interest from pUC19 DNA (*SstI* cleaved in the multiple cloning site near the *HincII*/*SmaI* junction). The 2.0 kb fragment was isolated from an agarose gel and ligated to pUC19, which had been digested with *SmaI* and *SstI*. This yielded the recombinant pVGT15, which has insert DNA bounded on one side by a *SmaI*/*AvaII* junction and on the other by a *HincII*/*SmaI* junction. The location and orientation of the structural genes for the SLTIIva A and B subunits are shown above the map. Arrows below the map indicate the direction and extent of DNA sequence analysis.

Table 3. Neutralization of VT in *E. coli* broth culture supernatants by porcine polyclonal antisera

<i>E. coli</i> strain	Neutralization titre*, with antiserum† against:		
	SLTI	SLTIIv	SLTII
H.I. 8	0	625	25
DH5 α (pVGT15)	0	625	25
412	0	3125	125
H19	3125	0	0
C600(933W)	0	25	3125

* The titre is the reciprocal of the highest dilution of serum which completely neutralized the effects of 10 CD₅₀ (CD, cytotoxic dose) of SLT preparations. Titres against the homologous type of SLT are shown in **bold type**.

† Antisera were prepared in pigs with purified SLTI and SLTIIv preparations and formalin-treated cultures of *E. coli* C600(933W) (SLTII⁺).

The probe hybridized with a single band from *HindIII* digests of total DNA, corresponding in size to the 4.5 kb *HindIII* insert of pVGT10, but did not hybridize with *HindIII*-digested plasmid DNA from this *E. coli* strain (see Fig. 2).

Nucleotide sequence analysis

The nucleotide sequence of the *slt* genes presented in Fig. 3 begins approximately 200 bases downstream of the *AvaII*/*SmaI* junction of pVGT15 and extends to the 3' end of the insert. Two open reading frames corresponding to the genes encoding the A and B subunits were located within this DNA fragment. Homology to the published DNA sequences and the predicted amino acid sequences of SLTs (Calderwood *et al.*, 1987; De Grandis *et al.*, 1987; Jackson *et al.*, 1987a; Weinstein *et al.*, 1988; Gyles *et al.*, 1988) is summarized in Table 4. The DNA sequence of the B subunit gene had the closest relationship (98%) to the B subunit region of *sltIIv* while the A subunit gene had a similar level of homology to the A subunit genes of *sltIIv* (70.6%) and *sltII* (69.5%). *sltI* had the least DNA sequence homology in both A and B subunit genes (58.8 and 64.3%, respectively).

A putative promoter was identified upstream of the A subunit initiation codon, with –35 and –10 regions with homology to the consensus hexamers of McClure (1985) (see Fig. 3). The putative promoter region of *sltIIva* had low homology to the corresponding regions of other *slt* operons (from 38.3% for *sltII* to 39.6% for *sltI*).

Putative ribosome-binding sites (Shine & Dalgarno,

Fig. 2. Hybridization experiments with *Hind*III-digested plasmid and total cellular DNA of *E. coli* strain H. I. 8. Photograph (a) is of a 0.8% agarose gel with DNA samples stained with ethidium bromide; photograph (b) is of an autoradiograph of a membrane following hybridization of transferred DNA samples with a ^{32}P -labelled 770 bp *Bgl*II-*Pst*I fragment from pVGT15. Lanes: 1, molecular mass markers; 2, *Hind*III-digested pVGT10 DNA; 3, caesium-chloride-purified plasmid DNA from *E. coli* H. I. 8 digested with *Hind*III; 4, total DNA from *E. coli* H. I. 8 digested with *Hind*III.

1974) for regions encoding the A and B subunits, respectively, are shown in Fig. 3. One of these occurs in the 15 nucleotide gap between the A and B subunits of *sltIIva*. The same number of nucleotides is present in the space between A and B subunit genes in the *sltIIv* operon (Weinstein *et al.*, 1988).

The region 3' to the termination codon of the B subunit of *sltIIva* contains two regions highly homologous to the

sltIIv sequence. The first includes a stretch of 82 nucleotides following the termination codon of the B subunit. This area of homology is interrupted by 38 nucleotides not found in *sltIIv*. Following this insertion another region of 119 nucleotides with high homology to *sltIIv* is present. Overall homology of the region sequenced 3' to the end of the B subunit was 74% for *sltIIv* as compared to 44.5% and 47% homology for *sltI* and *sltII*, respectively. Similar regions of high homology were not observed in the latter gene sequences.

A stretch of DNA with several features of a rho-independent termination signal (Brendel & Trifonov, 1984) was found downstream of the B subunit gene (see Fig. 3). These features include a GC-rich region in the 3' portion, a region of dyad symmetry with a high predicted free energy ($-17.4 \text{ kcal mol}^{-1}$; $-72.8 \text{ kJ mol}^{-1}$) for the 8 base stem structure, a T-rich stretch and the sequence TCTG at the 5' end of the region. However, the single nucleotide of the 'loop' and the absence of G nucleotides on the 3' side of the stem is somewhat anomalous.

Putative signal sequences (von Heijne, 1984, 1985) for A and B subunits were identified (Fig. 3) 22 and 19 amino acids from the N-terminus of the respective subunit polypeptides. Based on these data the processed A subunit consists of 297 amino acid residues and has a molecular mass of 33091 Da, while the processed B subunit consists of 68 amino acids and has a molecular mass of 7556 Da. *SLTIIv* is thought to contain the same number of amino acids in the respective processed A and B subunits (Weinstein *et al.*, 1988). A site susceptible to cleavage by trypsin was identified in the A polypeptide chain (position 250) which is bounded on each side by a cysteine residue. Tryptic cleavage could, therefore, result in the generation of A1 and A2 fragments.

The relationship of sltIIva to other slt genes

Three 17-base regions of the *sltIIva* A subunit sequence, one each in the 5' end, middle portion and 3' end, were selected at random to examine the relationship of the

Table 4. Homology comparison of the nucleotide and predicted amino acid sequence of the processed A and B subunits of the *sltIIva* genes

Homology is expressed as the percentage relatedness of sequences obtained using the ALIGN function of the MicroGenie computer program.

	Nucleotide sequence		Amino acid sequence	
	A subunit	B subunit	A subunit	B subunit
SLTIIv	70.6	98.0	72.4	97.1
SLTII	69.5	78.1	71.4	84.3
SLTI	58.8	64.3	56.2	62.3

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0      10      20      30      40      50      60      70      80
      +G +++ GA G C CG C AC GGGT G TGGT CGGGG CG GTCC +G TGGCGG ATT T
ATATGTGAGAGATTCTCTATTCTATAAAATATAAACCATATACCCGTAATCTTAAATAATTGATACTATTTCCGGAAG
-35
      90      100      110      120      130      140      150      160
GC + CATG G G TA CAG CGGA A GC TT CCTTC GCA C GT C GG CGAATCC TAC A
CTAGTGTGATCATTATCTGCTTTCTGTTCCTGATAAACAGGGAAGTAATACATTGTGCAGTCATTTCTGTGAGGGGAT
-10
      170      180      190      200      210      220      230      240
      (CC) (TC) (A)
CGCGCCAC C T T GCTCGCTTC G G C T TGT T CGCCGGGT TA ATTACCTTA A A
TTGTTTGAATAAAATTTCTGCTAAAAACATTCCGACITTCITGCGAGGTATTATTCTGGCGCTGCCGTTTCAGACTGAT

      250      260      270      280      290      300
A A ATG AAG TG G TG A A C G C G TA C G G T T
GGAGTGTTAATAT ATG CGA CAT ATA TTA TTA AAG CTG GTG TFG TTT TTT TGT GTT TGC TTG TCT
SD Met Arg His Ile Leu Leu Lys Leu Val Leu Phe Phe Cys Val Cys Leu Ser
Lys Cys Trp Ile Cys Leu Leu Leu Gly Phe
-22 -10

      310      320      330      340      350      360
G T C T C C G G A A C T G A T C A T A TC
TCA GCA TCT TAT GCA GAT GAG TTT ACT GTG GAT TTC TCT TCG CAA AAG AGC TAT GTT GAT
Ser Ala Ser Tyr Tyr Glu Phe Thr Val Asp Phe Ser Ser Gln Lys Ser Tyr Val Asp
Val Ser Gln Ile Ile Thr Thr Gln Ser
-1 +1 10

      370      380      390      400      410      420
G A C A A G C T A C AAT ATA TCT CAG GGT GGT
TCA TTG AAT AGT ATA AGG TCG GCA ATA TCC ACT CCA CTT GGA AAT ATA TCT CAG GGT GGT
Ser Leu Asn Ser Ile Arg Ser Ala Ile Ser Thr Pro Leu Gly Asn Ile Ser Gln Gly Gly
Thr Thr Glu His Ala
20 30

      430      440      450      460      470      480
ACA G A C T ACA C CCA GT T C G A GG A A C
GTT TCT GTT TCA GTA ATT AAT CAT GTT CTA GGC GGA AAC TAT ATA TCA TTG AAT GTT AGA
Val Ser Val Ser Val Ile Asn His Val Leu Gly Gly Asn Tyr Ile Ser Leu Asn Val Arg
Thr Thr Pro Pro Ser Val Gly Ile
40 50

      490      500      510      520      530      540
G GTT CAG C T G T C G T T A T
GGC CTT GAT CCA TAT AGC GAG AGA TTT AAC CAC CTC CGT TTA ATA ATG GAA CGG AAT AAC
Gly Leu Asp Pro Tyr Ser Glu Arg Phe Asn His Leu Arg Leu Ile Met Glu Arg Asn Asn
Val Gln Ile
60 70

      550      560      570      580      590      600
G T A G AC A CT C T A T
TTA TAT GTT GCA GGC TTT ATT AAT ACT GAA ACG AAT ATC TTT TAC AGA TTC TCC GAT TTC
Leu Tyr Val Ala Gly Phe Ile Asn Thr Glu Thr Asn Ile Phe Tyr Arg Phe Ser Asp Phe
Val Thr Thr
80 90

      610      620      630      640      650      660
G A T C G C A A A C
TCA CAT ATT TCA GTG CCT GAT GTG ATA ACT GTT TCC ATG ACG ACG GAC AGC AGT TAT TCA
Ser His Ile Ser Val Pro Asp Val Ile Thr Val Ser Met Thr Thr Asp Ser Ser Tyr Ser
Ala Leu Gyl Thr Ile
100 110

      670      680      690      700      710      720
A T C G A T G CG T C A A C A T C
TCA TTA CAG CGA ATC GCA GAT CTG GAA CGT ACA GGG ATG CAG ATT GGG CGT CAT TCA CTG
Ser Leu Gln Arg Ile Ala Asp Leu Glu Arg Thr Gly Met Gln Ile Gly Arg His Ser Leu
Thr Val Ala Ser
120 130

      730      740      750      760      770      780
TCA CG T T AA A A A A AT G A
GTT GGT TCA TAT CTG GAT TTA ATG GAG TTC AGA GGA CGT TCC ATG ACC CGC GCA TCA TCC
Val Gly Ser Tyr Leu Asp Leu Met Glu Phe Arg Gly Arg Ser Met Thr Arg Ala Ser Ser
Ser Ala
140 150

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790      800      810      820      830      840
  A G T      T C C      C T A      G      G
AGA GCT ATG CTG CGT TTT GTC ACA GTG ATA GCA GAA GCT CTG CGA TTC AGA CAA ATA CAG
Arg Ala Met Leu Arg Phe Val Thr Val Ile Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
      Val      Thr

160      170

850      860      870      880      890      900
A A A      T T A      A A T G      T G      G      C G G A C
CGG GGA TTC CGA CCG GCG CTG TCT GAG GCA TCT CCG CTT TAT ACA ATG ACG GCT CAG GAT
Arg Gly Phe Arg Pro Ala Leu Ser Glu Ala Ser Pro Leu Tyr Thr Met Thr Ala Gln Asp
      Glu      Leu      Thr Ala      Val      Pro Glu

180      190

910      920      930      940      950      960
  G      C T      G      C C      G      T C G
GTT GAC CTT ACC CTG AAC TGG GGA AGA ATA AGT AAT GTT CTT CCA GAG TAC AGA GGA GAG
Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu

200      210

970      980      990      1000      1010      1020
CT T C      G G G A      C      A A A A      A T      T C G
GAA GGG GTA AGA ATC GGT AGG ATA TCT TTT AAT AGT CTT TCT GCG ATT CTC GGA AGT GTT
Glu Gly Val Arg Ile Gly Arg Ile Ser Phe Asn Ser Leu Ser Ala Ile Leu Gly Ser Val
Ala      Val      Asn Ile      Thr

220      230

1030      1040      1050      1060      1070      1080
      (C)
  C T A G      +      T A      G GCG CG      T      C G      AT G      G G
GCG GTC ATC CTT AAT TGC CAC TCA ACC GGA AGT TAT TCA GTT CGT TCC GTG AGC CAA AAA
Ala Val Ile Leu Asn Cys His Ser Thr Gly Ser Tyr Ser Val Arg Ser Val Ser Gln Lys
      His Gln      Ala Arg      Ala      Asn Glu Glu

240      250

1090      1100      1110      1120      1130      1140
AGT C C      T      A AC C      C C TT A      A C      ACA A
CAG AAA ACA GAA TGC CAG ATT GTT GGA GAC AGG GCG GCC ATT AAA GTA AAT AAT GTT TTG
Gln Lys Thr Glu Cys Gln Ile Val Gly Asp Arg Ala Ala Ile Lys Val Asn Asn Val Leu
Ser Gln Pro      Thr      Pro Val      Ile      Thr

260      270

1150      1160      1170      1180      1190      1200
      AGT      GCA A G T C G C A A      T A      TC T A TA AC A T
TGG GAA GCG AAT ACA ATC GCT GCT TTA TTA AAT CGC AAG CCT CAG GAT CTT ACT GAA CCA
Trp Glu Ala Asn Thr Ile Ala Ala Leu Leu Asn Arg Lys Pro Gln Asp Leu Thr Glu Pro
      Ser      Ala      Phe      Ser      Ser      Tyr Thr Thr

280      290

1210      1220      1230      1240      1250      1260
GGT G      G A      A T A GA      T      A G      T CA T G G
AAC CAA TAA CAG GGG GTG AAT ATG AAG AAG ATG ATT ATT GCA GTT TTA TTC GGT CTC TTT
Asn Gln      SD      Met Lys Lys Met Ile Ile Ala Val Leu Phe Gly Leu Phe
Gly Glu      -19      Phe      -10

1270      1280      1290      1300      1310      1320
  T      G A      AA      T
TCT GCT AAT TCC ATG GCG GCG GAT TGT GCT GTA GGA AAA ATT GAG TTT TCC AAG TAT AAT
Ser Ala Asn Ser Met Ala Ala Asp Cys Ala Val Gly Lys Ile Glu Phe Ser Lys Tyr Asn
      Val      Ala      Lys

-1 +1      10

1330      1340      1350      1360      1370      1380
  A
GAG GAT GAT ACC TTT ACT GTG AAG GTG TCA GGA AGA GAA TAC TGG ACG AAC AGA TGG AAT
Glu Asp Asp Thr Phe Thr Val Lys Val Ser Gly Arg Glu Tyr Trp Thr Asn Arg Trp Asn
      Asn

20      30

1390      1400      1410      1420      1430      1440
TTG CAG CCA TTG TTA CAA AGT GCT CAG CTG ACA GGG ATG ACT GTA ACA ATC ATA TCT AAT
Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr Gly Met Thr Val Thr Ile Ile Ser Asn

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Fig. 3. Nucleotide sequence of the *sltIIva* operon from *E. coli* strain H.I.8. Numbers above the sequence indicate the nucleotide position and those below the sequence the amino acid residue position. The open reading frame for the *sltIIva* A subunit structural gene extends from nucleotide positions 254 to 1210 and that for the B subunit structural gene from nucleotide positions 1226 to 1486. The -35 and -10 regions of the proposed promoter are indicated. Shine-Dalgarno (SD) sequences are located upstream of the start codons for the respective A and B subunit sequences. Negative numbers are given to amino acids in the signal peptide while those in the processed polypeptides are positive. The proposed cleavage sites are located between amino acid residues -1 and +1. The sequence is compared with the published *sltIIv* nucleotide sequence (Weinstein *et al.*, 1988) from nucleotides 1 to 1715 of *sltIIv*. In the comparison the DNA sequence 5' to the initiation codon, the nucleotide and predicted amino acid sequence of structural genes for the A and B subunits, nucleotides in the gap between A and B subunits and the DNA sequence 3' to the termination codon of the gene for the B subunit were aligned separately with the corresponding regions of *sltIIv*. These data have been combined in the figure. Nucleotides from *sltIIv* which differ from those of *sltIIva* are placed above the *sltIIva* nucleotide sequence and amino acid residues which differ are placed below those of the *sltIIva* amino acid sequences. The plus signs above nucleotides indicate that they are present in *sltIIva* but not in *sltIIv* and parentheses indicate nucleotides present in *sltIIv* but not in *sltIIva*. A putative rho-independent terminator is located from residues 1680 to 1706; the inverted repeat present in this region is underlined.

Fig. 4. Hybridization of oligonucleotide probes complementary to the *sltIIva* A subunit gene with total cellular DNA of *E. coli* strains. Total DNA (5 µg per slot) of *E. coli* strains was transferred to nylon membranes with a slot-blot apparatus. *E. coli* strains: C600 *slt*⁻ (positions 1 and 8), H.I.8 (O128:B12) *sltIIva*⁺ (position 2), C600(933W) *sltII*⁺ (position 3), 412 (O139:K82) *sltIIv*⁺ (position 4),

sltIIva A subunit DNA sequence to that of other *slt* genes. The three synthetic oligonucleotide DNA probes (5'-TGTCACAGTGATAGCAG-3', 5'-GGCCTTG-ATCCATATAG-3', and 5'-GTCTTCAGCATCTT-ATG-3') selected each hybridized under highly stringent conditions with total DNA from *E. coli* strain H.I.8 but hybridized weakly or not at all with the total DNA from several other SLT-producing *E. coli* strains examined, including the K12 strain C600(933W)(SLTII), human pathogens H19 (O26:H11)(SLTI), E32511 (O157:H-)(VT2) and B2F1/3 (O91:H21)(VT2) and an oedema disease strain 412 (O139:K82)(SLTIIv) (Fig. 4).

E32511 (O157:H7) *vt2*⁺ (position 5), B2F1/3 (O91:H21) *vt2*⁺ (position 6) and H19 (O26:H11) *sltI*⁺ (position 7) are shown. Autoradiographs are shown of membranes which were hybridized with the following ³²P-labelled oligonucleotide probes: 5'-TGTCACAGTGATAGCAG-3' (A), 5'-GGCCTTGATCCATATAG-3' (B) and 5'-GTCTTCAGCATCTTATG-3' (C).

Discussion

In the comparison of the nucleotide sequences of the four *slt* genes, areas of high homology of *sltIIva* with *sltIIv* were readily apparent. The close relationship was most obvious in the nucleotide sequence encoding the B subunit and the region downstream of its termination codon. The interruption of this region of homology by a 38 nucleotide stretch in *sltIIva* suggests that recombination rather than simple base substitution has occurred in the evolution of these two toxin genes. The relationship among the regions encoding the A subunit and promoter region for the toxin genes is less clear and is more consistent with genetic divergence resulting from single base alterations.

Several reports in the literature suggest circumstances by which genetic recombination between *sltIIva* and *sltIIv* genes may have occurred. Firstly, the co-occurrence of SLT variants within single *E. coli* strains has been noted (Scotland *et al.*, 1985; Strockbine *et al.*, 1986) and provides the necessary situation for this event. In this regard, O'Brien *et al.* (1989) have recently suggested that homologous recombination between *sltI* and *sltII* or other bacteriophage genes may occur in *E. coli* strain 933(O157:H7). Genetic rearrangement such as this could clearly lead to variation in *slt* genes. Secondly, Kozlov *et al.* (1988) have reported that DNA with homology to an *E. coli* insertion sequence flanks the Shiga toxin gene of *S. dysenteriae*. Transposition may be another means by which gene transfer and recombination may have taken place in this toxin group.

Information from this study suggests that the *sltIIva* gene is located on the chromosome. Although we were unable to induce a bacteriophage or transfer VT from *E. coli* H.I.8 to *E. coli* C600, further study will be required to determine the nature of DNA sequences flanking the *sltIIva* gene.

While nucleotide sequence comparisons of *sltI*, *sltII* and *sltIIv* genes firmly establish differences among these genes, recent reports in the literature suggest that several additional types of SLT (VT) await characterization. Three *E. coli* strains which are reported to produce these toxins include E32511 (O157:H7) (Dickie *et al.*, 1989), which produces the 'VT2' prototype (Scotland *et al.*, 1985), B2F1/3 (O91:H21) (Oku *et al.*, 1989) and H.I.8 (O128:B12) (Gannon & Gyles, 1990). The DNA sequence data presented in this study support the contention that the SLT of *E. coli* strain H.I.8 does indeed represent a distinct type of the toxin. While we have previously shown that the VT of strain H.I.8 differs from other SLTs in heat lability and biological activity (Gannon & Gyles, 1990), a comparison of the *sltIIva* with other *slt* genes had not been made at the nucleotide sequence level. We were particularly interest-

ed in the relationship of *sltIIva* to *slt* genes from *E. coli* strains which may produce other types of SLT. In this study, three synthetic oligonucleotide DNA probes, complementary to the *sltIIva* A subunit gene, were used in hybridization studies to examine this relationship. Failure of these probes to hybridize strongly with DNA of other SLT-producing *E. coli* strains suggests that *sltIIva* differs from the *slt* genes examined. However, this does not imply that *sltIIva* or toxins more closely related to it do not occur in other *E. coli* strains. A comparison of *sltIIva* with genes of other O serogroup 128 *E. coli* strains, particularly those which fail to hybridize with VT1- or VT2-specific DNA probes (Smith *et al.*, 1987), would be of interest.

SLTs and Shiga toxin A subunits have been shown to possess N-glycosidase activity and inhibit protein synthesis by cleavage of the terminal portion of the 28S ribosomal RNA subunit (Endo *et al.*, 1988; Saxena *et al.*, 1989). This mechanism of action is shared by plant toxins such as ricin (Endo *et al.*, 1987). Homology of the amino acid sequence of the processed A subunit of SLTs with ricin A chain has been noted by several workers (De Grandis *et al.*, 1987; Calderwood *et al.*, 1987; Weinstein *et al.*, 1988) and recently, Hovde *et al.* (1988) have demonstrated, with oligonucleotide-directed mutagenesis, that the glutamic acid residue at position 167 of SLTI is necessary for full enzymic activity of the toxin subunit. A number of common amino acids were noted from positions 165 to 206 of the processed SLTIIva A subunit and 175 to 215 of the ricin A chain (see Fig. 5). This includes the glutamic acid residue mentioned above as well as several additional residues which are common to SLTI, SLTII and ricin. These residues are thought to occur in the enzymically active site or 'cleft' of the ricin A chain.

Recent studies have shown that SLTI and SLTII bind specifically to globotriosylceramide (Waddell *et al.*, 1988; Boyd & Lingwood, 1989) and that SLTIIv binds with high affinity to globotetraosylceramide (De Grandis *et al.*, 1989) and that these glycolipids, respectively, are probably the toxin receptors on cell surfaces. The high level of homology between the B subunits of SLTIIv and SLTIIva suggests that these toxins may have a common receptor which differs from that of SLTI and SLTII. This suggestion is supported by the low level of activity of SLTIIv and SLTIIva preparations against HeLa 229 cells relative to Vero cells. It will be of interest to see if differences in receptor specificity among SLTs result in different manifestations of disease in man and animals.

Finally, considerable debate exists with respect to the nomenclature of this group of *E. coli* toxins. Konowalchuk *et al.* (1977) first used the term 'Vero toxin' to describe the effects of certain *E. coli* broth culture

Fig. 5. A comparison of homologous amino acids in the A subunit of SLTIIva (positions 165–206) with the ricin A chain (positions 175–215) was conducted using the MicroGenie alignment program. Solid vertical lines indicate identity; dots indicate conservative amino acid substitutions. Conserved amino acids in the cleft of the ricin A chain crystal structure are those reported by Hovde *et al.* (1988). These are boxed where agreement occurs between the A subunit of SLTIIva and the ricin A chain. Gaps were introduced into both sequences to maximize the alignment.

supernatants on Vero cells. Continued use of this term in the literature is presumably in recognition of these pioneering efforts. O'Brien *et al.* (1982) and others have employed the term 'shiga-like toxin' to describe this group of *E. coli* toxins because of their relationship to Shiga toxin. They argue that if historical precedent is to be followed, then Shiga's discovery of the related toxin from *S. dysenteriae*, prior to the turn of the century, should be the precedent. Our use of the term SLTIIva is intended to indicate the close relationship of the toxin of strain H.I.8 to SLTIIv and to allow for simple designation of other variants which may be characterized. Thus other variants could be called SLTIIvb, c, etc. When a single system of nomenclature is agreed on, it will be possible to rename toxins as appropriate.

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