Cloning of Genes Determining the Production of Vero Cytotoxin by *Escherichia coli*

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Sequences encoding the production of a cytotoxin (VT) active on Vero cells were cloned in *Escherichia coli* K12 from a VT-determining phage that originated in *E. coli* strain H19 of serotype O26. H11. Subcloning resulted in the identification of a 2.5 kb fragment that still coded for VT production. Mutagenesis with transposon Tn1000 was used to map VT sequences and a 0.75 kb probe was developed. In colony hybridization tests with strains isolated from patients with haemolytic uraemic syndrome or diarrhoea, this probe derived from the H19 VT genes detected only some of the VT⁺ strains belonging to serogroup O157. A VT⁺ strain, E32511, serotype O157. H⁻, which was negative in colony hybridization was the source of another VT-determining phage from which VT sequences were cloned. Southern hybridization of the VT genes from E32511 with the H19 probe was negative under stringent conditions but there was weak homology under conditions of low stringency. These results indicate that there are differences in the VT genes of pathogenic *E. coli*.

INTRODUCTION

Certain strains of *Escherichia coli* that cause diarrhoea have been shown to produce a heat-labile cytotoxin active on Vero cells (Konowalchuk *et al.*, 1977; Scotland *et al.*, 1980*a*). The Vero cytotoxin (VT) was distinguishable from the heat-labile and heat-stable enterotoxins of *E. coli*. It has been proposed that VT is probably identical to Shiga toxin, the cytotoxin produced by strains of *Shigella dysenteriae* type 1 (O'Brien *et al.*, 1982, 1983*a, b*). Production of VT in *E. coli* has been associated with certain strains belonging to enteropathogenic serogroups that cause diarrhoea in infants and in which production of heat-labile or heat-stable enterotoxins has not been demonstrated. The occurrence of VT production in these strains is most common in serogroup O26 but VT⁺ strains belonging to other serogroups have been reported (Konowalchuk *et al.*, 1977; Wade *et al.*, 1979; Scotland *et al.*, 1980*a, b*).

Studies in Canada and the USA have demonstrated VT production by *E. coli* strains of serotype O157. H7 associated with cases of haemorrhagic colitis (Johnson *et al.*, 1983; O'Brien *et al.*, 1983*b*) and by strains of serogroups O26, O111, O113 and O157 isolated from patients suffering from haemolytic uraemic syndrome (HUS) (Karmali *et al.*, 1983*a, b*). The latter syndrome, principally seen in young children, is frequently associated with prodromal bloody diarrhoea. Several cases of HUS were reported in England and Wales during 1983 and VT-producing *E. coli* O157. H7 were isolated from some cases (Gulley, 1984; Smith *et al.*, 1984).

VT-producing *E. coli* of serogroups O138, O139 and O141 have also been associated with enteric infections in pigs, particularly with post-weaning diarrhoea and oedema disease (Konowalchuk *et al.*, 1977; Kashiwazaki *et al.*, 1980, 1981; Williams Smith *et al.*, 1983). However, evidence from all these workers indicated that the VT from porcine strains was antigenically distinct from that produced by human *E. coli*.

Abbreviations: HUS, haemolytic uraemic syndrome; VT, Vero cytotoxin.

Studies on the genetics of VT production showed that the property could be transferred from a strain of *E. coli* O26.H11 (strain H19) to *E. coli* K12 (Williams Smith & Linggood, 1971; Scotland *et al.*, 1980a). However, there was no evidence for plasmid involvement, and subsequently the genes encoding VT in H19 were shown to be on a bacteriophage (Scotland *et al.*, 1983; Williams Smith *et al.*, 1983). The latter authors also identified VT-carrying phages in a second O26 strain and in two strains of serogroup O128. Phages that determine VT production have also been detected in two *E. coli* O157 strains isolated from cases of HUS and bloody diarrhoea in Britain (Smith *et al.*, 1984), and production of Shiga-like toxin has been shown to be phage mediated in a strain of O157.H7 isolated during an outbreak of haemorrhagic colitis (O'Brien *et al.*, 1984). In contrast to this situation in human strains, a bacteriophage has not been implicated in the mediation of VT production in a porcine *E. coli* strain (Williams Smith *et al.*, 1983).

Preliminary experiments showed that a VT-determining phage from strain H19, purified from *E. coli* K12, contained DNA with a molecular weight of approximately 30×10^6 (Scotland *et al.*, 1983). The present study was undertaken to extend the physical characterization of the phage genome and, particularly, to locate the genes determining production of VT by molecular cloning and transposon mutagenesis. This information was then applied to the development of specific genetic probes for the detection of VT⁺ *E. coli* associated with HUS and other diseases in which VT production has been implicated.

METHODS

Strains, plasmids and bacteriophages. *E. coli* K12 AB1157 (Bachmann, 1972) was used for propagation of the bacteriophages coding for VT production. The strains used in the mating and cloning experiments were AB1157, HB101 (Maniatis *et al.*, 1982) and 14R519 (*E. coli* K12 Lac⁻ Pro⁻ Trp⁻ His⁻ Nal^r). Wild-type *E. coli* strains used in hybridization experiments are listed in Table 1. The vectors used for cloning DNA fragments were pBR322, which determines ampicillin and tetracycline resistance (ApTc), pACYC184, chloramphenicol and tetracycline resistance (CmTc), and pACYC177, ampicillin and kanamycin resistance (ApKm). The plasmid F⁺*pro lac*::Tn5 (Jorgensen *et al.*, 1979) was used in transposon mutagenesis experiments. Bacteriophages coding for VT production were described previously (Scotland *et al.*, 1983; Smith *et al.*, 1984); the VT phages originated in *E. coli* strain H19, serotype O26.H11 and strain E32511, serotype O157.H⁻.

Tests for VT production. Large numbers of colonies were screened for VT production by a rapid method adapted from that used to detect heat-labile enterotoxin. Monolayers of Vero cells grown in medium 199 with Earle's salts were prepared in microtitre tissue culture plates. Each bacterial colony was grown in 0.5 ml syncase/glucose medium without shaking at 37 °C for 24 h. A sample of culture (50 µl) was added to a monolayer of Vero cells and after 10 min the culture was removed and the monolayer washed with PBS (0.15 M-NaCl, 0.15 M-sodium phosphate, pH 7.2). Growth medium containing gentamicin (40 µg ml⁻¹), streptomycin (100 µg ml⁻¹) and penicillin (100 IU ml⁻¹) was added and the plate resealed and incubated at 37 °C for 3–4 d. After fixing with methanol and staining with Giemsa stain, the monolayer was examined for any cytotoxic effect.

VT production was confirmed using sterile culture supernatants by the method described previously (Scotland *et al.*, 1980a).

Propagation and preparation of phages. VT phages from strains H19 and E32511 were propagated on the indicator strain AB1157 grown in L broth containing 5 mM-MgSO₄. Phage was precipitated with polyethylene glycol 6000 (10%, w/v) and the concentrated phage was purified by banding in a caesium chloride step gradient. The resulting phage stock contained between 10¹¹ and 10¹² p.f.u. ml⁻¹. Phage DNA was extracted with phenol and dialysed against 10 mM-Tris/1 mM-EDTA, pH 8.

Preparation of plasmid DNA. Purified DNA of plasmid vectors and recombinant plasmids was prepared from 500 ml cultures by polyethylene glycol precipitation and caesium chloride density gradient centrifugation (Humphreys *et al.*, 1975). For rapid screening, DNA of recombinant plasmids was extracted by the method of Birnboim & Doly (1979).

Restriction endonuclease treatment and recombinant DNA techniques. Samples of phage or plasmid DNA were digested with restriction enzyme under the conditions suggested by the manufacturers (BRL or Boehringer). Where appropriate, digested source and vector DNAs were mixed and incubated with T4 DNA ligase at 10–15 °C overnight. The mixtures were used to transform *E. coli* K12 (Dagert & Ehrlich, 1979). Transformants were selected according to the drug resistance markers of the vector plasmid (see above) and those carrying hybrid plasmids were identified by insertional inactivation of these markers.

Isolation of mutants no longer coding for VT production. Transposon mutagenesis was used to isolate plasmid mutants that no longer coded for VT production. The F prime factor F⁺*pro lac*::Tn5 carries the $\gamma\delta$ sequence

designated Tn1000 (Guyer, 1978). This plasmid was transferred into *E. coli* K12 carrying NTP703, a recombinant plasmid coding for VT production (see Results). Strains carrying NTP703, which determines tetracycline resistance, and F'*pro lac*::Tn5, which codes for kanamycin resistance, were mated for 1 h at 37 °C with AB1157 with selection for tetracycline resistance (10 µg ml⁻¹). Transconjugants were examined for plasmid DNA and the molecular weights compared with that of NTP703. Strains carrying NTP703 with Tn1000 insertions were tested for VT production. Plasmid DNA preparations from these strains were used to transform *E. coli* 14R519 and transformant colonies were examined by agarose gel electrophoresis to check for the presence of NTP703::Tn1000 only.

Agarose gel electrophoresis. Initial examination of plasmid DNA was on vertical 0.6% (w/v) agarose gels run in TB buffer containing 89 mM-Tris base, 89 mM-boric acid and 2.5 mM-EDTA (Willshaw *et al.*, 1979). The products of restriction enzyme digestion were separated on vertical 1% (w/v) agarose gels run for 16 h in TA buffer (pH 7.9) containing 40 mM-Tris base, 5 mM-sodium acetate and 1 mM-EDTA. Preparative electrophoresis to isolate specific DNA fragments for radioactive labelling was done similarly using wide slots to accommodate large sample volumes. For the analysis of DNA fragments smaller than 750 bp, separation was on gels containing 3% (w/v) acrylamide and 0.5% (w/v) agarose run in TB buffer at 70 V for 3 to 4 h (Peacock & Dingman, 1968). Standards for the determination of molecular weights of restriction fragments were either λ phage DNA treated with *Hind*III and *Eco*RI (Southern, 1979) or DNA of pBR322 digested with *Hinf*I (Maniatis *et al.*, 1982), depending on the size range of fragments separated. Transfer of DNA fragments from gels to nitrocellulose (Schleicher & Schüll BA85) was by the method of Southern (1975).

Preparation of filters for colony hybridization. Broth cultures of strains were spotted on BA85 nitrocellulose filter discs (diam. 82 mm). The filters were incubated overnight on MacConkey agar plates at 37 °C and then prepared for hybridization by the method of Maniatis *et al.* (1982).

DNA hybridization. Restriction fragments for use as hybridization probes were excised under long wavelength UV from preparative agarose gels and recovered by electroelution in TA buffer. The DNA solution was extracted once with phenol (equilibrated with TE buffer containing 0.01 M-Tris HCl, pH 8, and 1 mM-EDTA) and several times with *n*-butanol before precipitation with ethanol. DNA eluted from several gels was pooled and dissolved in 30–50 µl TE buffer. DNA fragments were radioactively labelled by nick translation (Maniatis *et al.*, 1982) using deoxyadenosine 5'- α -[³⁵S]thiotriphosphate (New England Nuclear).

The conditions for hybridization of denatured radio-labelled probe to DNA immobilized on nitrocellulose filters were as follows.

(1) Hybridization at 'high' stringency was carried out in a mixture containing 50% (v/v) formamide, 5 $\times$ SSPE (1 $\times$ SSPE, pH 7.4, is 0.18 M-NaCl, 10 mM-NaH₂PO₄ and 1 mM-EDTA), 0.1% each of Ficoll (MW 400000), polyvinylpyrrolidone, bovine serum albumin and SDS, 0.01 M-dithiothreitol and 100 µg denatured salmon sperm DNA ml⁻¹. After washing for 2 h in 50 mM-Tris/HCl (pH 8.0) containing 1 M-NaCl, 1 mM-EDTA and 0.1% SDS, nitrocellulose filters were incubated for 4 h in hybridization solution without labelled probe. They were then transferred to 20–25 ml fresh hybridization solution containing 10⁶ to 10⁷ c.p.m. heat-denatured probe DNA and incubated for 16 h. All stages were at 42 °C. Washing of the filters was in (i) four changes (10 min each) of 2 $\times$ SSC containing 0.1% SDS at room temperature (1 $\times$ SSC is 0.15 M-NaCl and 0.015 M-trisodium citrate, pH 7.0); (ii) 1 $\times$ SSC/0.1% SDS at 68 °C for 2 h; (iii) 0.2 $\times$ SSC/0.1% SDS for 1 h at 68 °C. Filters were allowed to dry at room temperature and exposed to Kodak XAR X-ray film at -70 °C for times varying between 7 and 40 h.

(2) Hybridization at lower stringency was carried out as described above except that the hybridization mixture contained 25% (v/v) formamide and the temperature was 37 °C (Moseley *et al.*, 1983). Washing was in 5 $\times$ SSC/0.1% SDS for 45 min at 54 °C followed by 30 s at room temperature in 2 $\times$ SSC.

RESULTS AND DISCUSSION

Cloning of phage DNA encoding VT and location of VT sequences

Unsuccessful attempts were made to clone VT sequences from complete digests of phage H19 (ϕ H19) using the restriction enzymes *Hind*III, *Bam*HI, *Pst*I, *Sal*I, *Xho*I, *Cl*aI, *Eco*RI, *Sma*I and *Bgl*II. However, a recombinant plasmid encoding VT was obtained from a digest of ϕ H19 DNA with *Hind*III plus *Xho*I that had been ligated with similarly cleaved pACYC177. This plasmid, NTP702 (Fig. 1a), had a molecular size of about 12 kb and contained a *Hind*III fragment of 3.2 kb and a *Xho*I-*Hind*III fragment of 5.1 kb.

To determine the approximate position of VT sequences, *Pst*I fragments of NTP702 were ligated with *Pst*I-cleaved pBR322. In two of three VT⁺ transformants obtained (20 tested) a 10.8 kb hybrid plasmid was present, the structure of which is shown in Fig. 1(b) (NTP703). The plasmid contained *Pst*I fragments of 6.3 kb and 0.45 kb from NTP702; the latter fragment did not contain a *Bam*HI site, indicating that in forming NTP703 the *Pst*I fragment of NTP702

Fig. 1. Partial restriction maps of DNA cloned from the H19 VT phage. Sites for restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; P, *Pst*I; X, *Xho*I. The map of NTP702 (a) includes a region of the vector plasmid pACYC177 (solid line) since this was retained in the formation of NTP703 (b). A length of NTP702 between the *Eco*RI and *Xho*I sites of the phage DNA has not been shown in full, and *Hinc*II sites are not included on this map. The map of NTP703 shows the sites of *Tn1000* insertion (arrowed) in the numbered plasmid mutants: +, no effect on VT production; —, loss of VT expression. The precise location of *Tn1000* in vector sequences in mutants 8 and 9 was not determined. The *Eco*RI fragment present in NTP706 (c) was derived from a *Hae*III fragment as described in the text.

lying between the two cloned fragments had been deleted (compare Figs 1a and 1b). A 5.4 kb *Bam*HI fragment of NTP702 (Fig. 1a) that is contained in NTP703 was cloned separately into pACYC184 to yield NTP704 (not shown); this plasmid coded for VT production and was used in some subsequent manipulations as indicated.

Further subcloning of VT sequences was carried out after the location of these had been suggested by mutagenesis and hybridization studies. A series of mutants of the plasmid NTP703 carrying *Tn1000* was generated as described in Methods. Of 19 strains tested for VT production 13 were VT⁺ and 6 were VT⁻. With one exception these strains carried a plasmid approximately 6 kb larger than NTP703, indicating that insertion of *Tn1000* into NTP703 had occurred.

DNA of all the NTP703::*Tn1000* mutants that no longer encoded VT was digested with *Hind*III or *Bam*HI and the fragment patterns compared with that of NTP703 and four NTP703::*Tn1000* derivatives that still specified VT. From examination of these results in conjunction with the known restriction map of *Tn1000* (Guyer, 1978), it was possible to map the insertion sites of the transposon on NTP703. These results are shown in Fig. 1(b). Of the four NTP703::*Tn1000* derivatives that still coded for VT production, two carried the transposon within vector DNA. In the other mutants, 2 and 10, *Tn1000* was inserted in phage DNA sequences as shown in Fig. 1(b). The sites of *Tn1000* insertion in NTP703 that resulted in loss of the capacity to encode VT production were clustered around the *Hind*III site in the cloned phage DNA. In four VT⁻ mutants, 6, 7, 16 and 19, *Tn1000* insertion had occurred to the left of this *Hind*III site within a 3.2 kb *Hind*III fragment of NTP703, whereas in mutants 5 and 17 the transposon was located to the right of the *Hind*III site. The *Tn1000* insertion sites spanned a region of about 0.6 kb.

Restriction mapping showed that this *Hind*III site in NTP703 and NTP704 was within a *Hinc*II fragment of 0.75 kb (Fig. 1b). The location of the *Tn1000* insertion was confirmed by examining *Hinc*II digests of the NTP703 mutants for the presence of this fragment. Control digests of the VT-encoding mutants 2 and 8 indicated that there were no similarly sized fragments arising from *Tn1000*. Of the mutants that had lost the ability to code for VT production, 5, 6, 7 and 17 did not have a 0.75 kb *Hinc*II fragment detectable visually on a gel or

by Southern hybridization of the blotted gel to isolated radioactively-labelled 0.75 kb *HincII* fragment. The VT non-encoding mutants 16 and 19 retained this *HincII* fragment, confirming that these insertions were outside the fragment.

These results indicated that the 0.75 kb *HincII* fragment might be a useful probe for VT sequences (see next section). It formed part of a region associated with VT production although the extent of adjacent DNA also involved was not known. The *HincII* fragment was cloned into *HincII*-cleaved pACYC177 but, as expected from the mutagenesis results, an *E. coli* K12 strain carrying the hybrid plasmid (NTP705, not shown) did not produce VT.

Restriction mapping of NTP703 failed to indicate any fragments that could be readily cloned which were likely to contain all the VT sequences. A *BamHI*-*BglII* fragment (2.2 kb; Fig. 1*b*) that spanned the region of interest was used in hybridization with DNA of NTP704 digested with various restriction enzymes. A *HaeIII* fragment of 2.5 kb hybridized with the *BamHI*-*BglII* fragment and was cloned from a digest of NTP704 by ligation with *EcoRI*-treated DNA of pACYC184 that had been rendered flush-ended (Maniatis *et al.*, 1982). Of 100 strains carrying recombinant plasmids, two were VT⁺ and carried identical 6.6 kb plasmids (NTP706; Fig. 1*c*). This is the smallest derivative obtained so far that codes for VT synthesis.

Further mapping experiments, together with the examination of plasmid-coded polypeptides in minicells are required to identify the regions coding for the subunits of the toxin. The Shiga-like toxins of *E. coli* strains H30 and 933 have been reported to have the same A and B subunit structure as Shiga toxin (O'Brien *et al.*, 1983*a*), although minor differences between Shiga toxin and the H30 Shiga-like toxin have been observed (O'Brien & LaVeck, 1983).

Application of VT probe sequences

Specificity of the probe. The specificity of the 0.75 kb *HincII* fragment as a probe for VT sequences was checked by hybridization to DNA of ϕ H19, NTP703, NTP705, pBR322, pACYC177, phage λ and NTP104, a plasmid of 63 kb determining heat-labile enterotoxin production. Restriction enzyme-generated fragments of phage or plasmid DNA were separated on agarose gels, transferred to nitrocellulose and hybridized with ³⁵S-labelled probe in the presence of 50% (v/v) formamide (see Methods). There was strong hybridization to the homologous unlabelled *HincII* fragment in digests of NTP703 and NTP705 but no vector sequences of pACYC177 or pBR322 were detected. Hybridization of the 0.75 kb *HincII* fragment to *HindIII*-digested ϕ H19 DNA detected homology with two *HindIII* fragments (3.2 kb and 7.0 kb), as would be predicted from the presence of a *HindIII* site in the probe sequence. There was no homology with fragments of control plasmid DNA of NTP104 or to phage λ . However, it appeared that some of the DNA cloned in NTP703 did have homology with λ DNA since in preliminary experiments, in which the 5.4 kb *BamHI* fragment of NTP703 (Fig. 1*b*) was used as a probe, a 6.6 kb *HindIII* fragment of λ hybridized.

Detection of VT⁺ E. coli. A panel of wild-type and K12 strains of *E. coli* was selected for colony hybridization tests with the radioactively labelled 0.75 kb *HincII* probe fragment. These experiments were done under conditions of high stringency. The wild-type *E. coli* comprised VT⁺ and VT⁻ O26.H11 strains including H19, *E. coli* of serotype O157.H7 or O157.H⁻ which produced VT and were associated with cases of HUS or bloody diarrhoea in Britain, and VT⁻ O157.H45 strains. The K12 strain used was AB1157; this was either plasmid-free or carried ϕ H19 or NTP705. Results of the hybridization tests are listed in Table 1.

There was clear distinction between VT⁺ and VT⁻ O26.H11 strains and between AB1157 and the same strain carrying the H19 VT phage or NTP705. Hybridization of this probe to VT⁺ O157 *E. coli* differentiated two classes. Strains E30228 and E30480 were detected as VT⁺ by this probe whereas E30979 and E32511 were probe negative. This indicated that there are differences in the VT genes of the O157 strains. Strains of serogroup O157 that did not produce VT were negative in tests with the 0.75 kb *HincII* probe.

Strain E32511, which was probe negative in colony hybridization tests, was selected for further study since VT production in this strain was shown to be phage determined (Smith *et al.*, 1984). DNA, prepared from E32511 VT phage (ϕ E32511), was digested with *HincII*. After transfer to nitrocellulose the fragments were tested for hybridization with the 0.75 kb H19

Table 1. *Testing of wild-type and K12 strains of E. coli by colony hybridization*

Strain no.	Serotype	VT production*	Hybridization†
H19	O26.H11	+	+
E9040	O26.H11	+	+
E9192	O26.H11	—	—
E7795	O26.H11	—	—
E30228	O157.H7	+	+
E30480	O157.H7	+	+
E30979	O157.H7	+	—
E32511	O157.H ⁻	+	—
E9818	O157.H45	—	—
E10376	O157.H45	—	—
K12 (H19 VT phage)		+	+
K12 (NTP705)		—	+
K12		—	—

* Assayed against Vero cells as described in Methods.

† With a 0.75 kb H19 *HincII* probe.

probe. Under conditions of high stringency no homology was detected. However, when lower stringency was used weak binding was observed with a 2 kb *HincII* fragment but no homology was detected at a position on the blot corresponding to 0.75 kb.

These results indicate that there is heterogeneity in the VT sequences of the O157 strains and this may reflect differences in the VTs encoded by these genes. These observations can be compared to the studies with the ST_{Ia} (or ST_{A1}) and ST_{Ib} (or ST_{A2}) genes (De Wilde *et al.*, 1981; Moseley *et al.*, 1983). The two ST genes have approximately 70% homology but do not hybridize under stringent conditions. However, the two ST toxins have a close structural and immunological similarity.

Cloning of VT sequences from the E32511 VT phage

DNA of ϕ E32511 was digested with *EcoRI* and fragments were ligated with similarly cleaved pACYC184. Of 50 transformants tested for VT production four were VT⁺. Three of these four strains carried recombinant plasmids with a molecular size of 8.6 kb. Digestion with *EcoRI* showed that the 8.6 kb plasmids carried inserts of 4.7 kb; one of these plasmids was designated NTP707. Hybridization of *HincII*-digested NTP707 with the 0.75 kb H19 probe showed that a 2 kb *HincII* fragment within the 4.7 kb *EcoRI* fragment showed weak homology under conditions of low stringency. This confirmed the results obtained with the total DNA of ϕ E32511.

The 2 kb *HincII* fragment of NTP707 and the 0.75 kb *HincII* probe derived from NTP705 are being used for hybridization to test strains of *E. coli* and *Shigella* and also to detect VT⁺ organisms in faecal specimens isolated from cases of HUS. Details of these experiments will be reported separately. The use of hybridization for testing strains present in faecal samples is particularly useful in cases where the proportion of VT⁺ organisms is low (Karmali *et al.*, 1983*b*). This approach, together with toxin testing and serotyping, should provide valuable information on the role of *E. coli* producing Vero toxins in the pathogenesis of HUS and bloody diarrhoea.

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