Cloning in *Escherichia coli* of the Enterotoxin Gene from *Clostridium perfringens* Type A

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A 26 bp DNA probe has been constructed with minimal degeneracy to the protein sequence for *Clostridium perfringens* enterotoxin. The probe has been hybridized against a 6–10 kb chromosomal bank from *C. perfringens* 8239, prepared as a HindIII partial digest in pHG165. From this survey a clone has been identified containing a 6.8 kb DNA insert with strong hybridization to the probe. Direct plasmid sequencing has identified a translational reading frame within this clone which correlates with the known protein sequence for the type A enterotoxin. DNA sequences 5' to this open reading frame and containing the putative transcriptional control regions show areas of significant homology with regions upstream from the ATG codon of the tetanus toxin gene.

INTRODUCTION

*Clostridium perfringens* is a Gram-positive, spore-forming, anaerobic rod. It is a normal soil inhabitant and is divided into five serotypes (A–E), of which only types A, C and D are associated with disease in man. The classic mild type of *C. perfringens* food poisoning is caused by type A. It is recognized as one of the major causes of food-poisoning outbreaks principally involving meat and meat products, although other foods are occasionally implicated (Bryan, 1969; Duncan, 1970; Genigeorgis, 1975). Hatheway *et al.* (1980) have published a review of epidemiological aspects of *C. perfringens* food-borne illness.

Foods involved in these outbreaks are generally thought to contain high numbers of vegetative cells (10^6–10^7 g^-1), some of which survive passage through the stomach and sporulate in the intestine. During sporulation an associated enterotoxin is formed and it is clear that all of the food poisoning symptoms correlate with the presence of this enterotoxin molecule (Stark & Duncan, 1971). The enterotoxin has long been thought to be a structural part of the spore coat (Frieben & Duncan, 1973). However, a recent study by Ryu & Labbe (1989) and previous work by Goldner *et al.* (1986) and Granum *et al.* (1984) suggest that enterotoxin gene expression may not be specific to sporulation. Principally this stems from the detection of enterotoxin in non-sporulating bacterial cultures. Despite substantial efforts, biochemical approaches have so far failed to resolve this and other aspects of enterotoxin production. With these aspects in view, we describe here the cloning of the *C. perfringens* enterotoxin gene into *Escherichia coli* and, in particular, present the sequence 5' to the structural gene which contains the putative genetic control regions for developmental expression.

METHODS

Production and purification of *C. perfringens* DNA. Chromosomal DNA was extracted from *C. perfringens* 8239 using the method of Okita *et al.* (1981) modified to include an acetone pre-wash of cells (Heath *et al.*, 1986). This procedure was adopted following difficulties in achieving lysis and is detailed below.

Cells were grown up overnight in 500 ml Reinforced Clostridial Medium (Oxoid) in Duran bottles within an anaerobic jar. For each extraction, 100 ml of culture was used with the remainder of the culture being stored at
Cells were harvested by centrifugation (10 000 g for 7 min at 4 °C). The cells were washed with 50 ml ice-cold buffer (50 mm-Tris/HCl, pH 7.2) and collected by centrifugation. The pellet was resuspended in 50 ml ice-cold acetone and kept on ice for 5 min. Cells were recovered by centrifugation and residual acetone removed from the pellet by evaporation under vacuum. The pellet was resuspended in 25 ml TE buffer (10 mm-Tris/HCl, pH 7.9, 0.1 mm-EDTA). Lysozyme (Sigma) was added to a final concentration of 5 mg ml⁻¹ and the suspension incubated at 37 °C. At 30 min intervals, 1 ml was removed and SDS added to a final concentration of 1 % (w/v). When complete clearing of the suspension occurred, SDS was added to the remaining bulk of the material, again to a final concentration of 1 % (w/v). RNAase (Sigma) [stock (10 mg ml⁻¹) preheated for 15 min at 80 °C and cooled slowly to room temperature] was added to a final concentration of 200 μg ml⁻¹. Incubation with RNAase was for 3 h at 37 °C, after which time proteinase K (Sigma) was added to a final concentration of 50 μg ml⁻¹ (stock solution 5 mg ml⁻¹). Incubation was continued for a further 30 min at 37 °C, after which 1 M-Tris/HCl, pH 9.0, was added to make a final concentration of 50 mM. An equal volume of STE-saturated phenol (STE: 10 mM-Tris/HCl, pH 7.9, 10 mM-NaCl, 1 mM-EDTA) was subsequently added and the mixture agitated gently on ice for 20 min. Following centrifugation (10 000 g for 10 min at 4 °C), the aqueous layer was withdrawn using a wide-bore plastic pipette. DNA was collected at the end of a glass rod after 2 vols ethanol (95%, w/v; −20 °C) had been layered over the extract. This DNA was redissolved overnight in 10 ml ice-cold 50 mM-Tris/HCl, pH 7.9. A further extraction was made with STE-saturated phenol followed by two extractions with CHCl₃. The DNA was then ethanol precipitated and resuspended in 2 ml TE buffer.

Production of a gene library in E. coli. Fragments of clostridial DNA in the desired size range (6–10 kb) were prepared by partial digestion with the restriction enzyme HindIII and cut out of a 0–8% (w/v) preparative agarose gel. DNA fragments were recovered from the agarose by electroelution into TAE buffer (80 mM-Tris/acetate, pH 7.8, 19 mM-EDTA) and purified by ion-exchange through a NACS 52 PREPACK (Gibco BRL). The purified DNA was ligated into the HindIII site of the multiple cloning site vector pHG165 (Stewart et al., 1986). The method of plasmid sequencing using AMV reverse transcriptase (Boehringer) was essentially that of Williams et al. (1986). The Klenow sequencing reactions were as described by Marusyk & Sergeant (1980) and Sergeant (1980) or by a miniprep procedure suitable for providing sequencing-grade DNA (Cannon et al., 1985). For both Klenow and reverse transcriptase, DNA was denatured for 5 min at room temperature using 4 μl plasmid DNA (0.5–1.0 μg) and 1 μl 2 m-NaOH. Annealing was accomplished by the addition of 1 μl primer (52 ng μl⁻¹), 1.5 μl 3 M-sodium acetate pH 4.6, 1.5 μl H₂O. The annealing reaction mixture was placed on a Millipore VSWP 0.025 pm automated device. The probe (2.5 μg) was labelled at the 5' end with [γ⁻³²P]ATP in a final volume of 25 μl (Maniatis et al., 1982). Unincorporated ATP was removed from the labelled probe by passage through a disposable prepacked Sephadex G25 column (Pharmacia LKB) pre-equilibrated with TE buffer.

Hybridization was overnight at 42 °C. Membranes were subsequently washed twice in 6 x SSC, 0.1% SDS at 42 °C for 30 min and once in 2 x SSC, 0.1% SDS for 30 min at 42 °C. Southern analyses were performed as described by Maniatis et al. (1982).

Plasmid sequencing. Plasmid DNA templates were provided either by CsCl purification (Guerry et al., 1973) or by a miniprep procedure suitable for providing sequencing-grade DNA (Cannon et al., 1985).

RESULTS AND DISCUSSION

Cloning the enterotoxin gene

A cloning strategy based on the synthesis of a DNA probe for hybridization against a genomic bank was possible because the protein sequence for the C. perfringens enterotoxin had been published previously (Granum, 1983). Fig. 1 shows the rationale for selection of the probe. Amino acids 9 through 17 provide the peptide sequence with minimal translational degeneracy. Nevertheless, when reverse-translated this region would predict a 26 bp primer with 512-fold
Enterotoxin clone

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Fig. 1. Selection of a gene probe for the C. perfringens enterotoxin. Amino acids 9 through 17 have a codon potential shown in DNA sequence (1) with the complementary sequence in (2). The 512-fold degeneracy of sequence (2) was reduced to fourfold degeneracy in sequence (3) by applying the convention that G-T interactions are neutral in terms of base pairing. Sequence (3) was used as a DNA probe and sequence (5) shows the actual codon usage for this region of the enterotoxin gene. Sequence (4) was used as the first plasmid sequencing primer.

degeneracy, a feature we have previously found to present significant problems in screening against false positives. The degeneracy of the probe was reduced, therefore, by applying the convention that G-T interactions are neutral in terms of base pairing (M. Suissa, personal communication). Using this convention a probe with only fourfold degeneracy was synthesized (Fig. 1).

There are no detectable plasmids in C. perfringens 8239 (data not shown); consequently, the DNA isolated was genomic. This DNA was resistant to digestion with the restriction enzymes Sau3A (GATC) and BamHI (GGATCC), both of which are inactive if their recognition sequence contains 5-methylcytosine. The DNA was, however, restricted by the Sau3A isoschizomer MboI, which is sensitive to adenosine methylation but not to 5-methylcytosine. These findings suggest the presence of a restriction-modification system in C. perfringens for which the core palindrome is GATC and for which methylation of the cytosine acts as a means of host protection. A restriction enzyme CpfI (GATC) has been previously identified in C. perfringens (R. Hansen, unpublished observations), but no information on an accompanying methylase has been reported. Chromosomal DNA in the 6–10 kb size range, from a partial HindIII digest, was cloned into the E. coli multiple cloning site vector pHG165 (Stewart et al., 1986), and used to transform E. coli DH5 alpha. Four thousand ampicillin-resistant colonies were screened for hybridization with the toxin probe as described in Methods. A single colony which gave a positive reaction against the probe was identified.

Plasmid DNA isolated from this clone was characterized by digestion with several restriction enzymes (Fig. 2). The results indicated that more than one recombinant plasmid was present within the clone.

These plasmids were extremely similar in size, as shown by the presence of only two bands, representing open circular and closed circular DNA, in track 3 of Fig. 2. Furthermore, apparently single linear bands were obtained after BamHI, Sall and SmaI digestion, reflecting both the absence of these sites within each of the three cloned inserts (linear DNA results from cleavage within the pHG165 multiple cloning site), and the very close similarity of insert sizes. One plasmid construct did show a significantly different copy number, as shown by bands of
Fig. 2. Restriction enzyme analysis of plasmid DNA isolated from an *E. coli* clone providing positive hybridization to a *C. perfringens* enterotoxin probe. Tracks 1 and 2, λcl857S7 cut with HindIII, 1 µg and 2 µg respectively; track 3, uncut plasmid; track 4, plasmid cut with *Bam*H1; track 5, plasmid cut with HindIII; track 6, plasmid cut with *Eco*RI; track 7, plasmid cut with *Sal*I; track 8, plasmid cut with *Pst*I; track 9, plasmid cut with *Sma*I.

Fig. 3. An enterotoxin-positive *E. coli* recombinant plasmid analysed by HindIII digestion and Southern hybridization. Track A, λcl857S7 digested with HindIII to provide molecular size markers; track B, a HindIII-digested single plasmid construct isolated from a *C. perfringens* bank; track C, Southern hybridization of track B with a 26 bp *C. perfringens* enterotoxin DNA probe.

mixed intensity in tracks 5, 6 and 8 which, at a glance, look similar to a partial reaction. In fact, the bands in these tracks do add up to the equivalent of three times the linear size of 9.2 kb.

The presence of multiple plasmids in the initial clone was confirmed by retransformation and selection for clones containing single plasmid derivatives. Southern hybridization in conjunction with the 26 bp toxin probe was used to identify which of the HindIII-digested plasmids contained a DNA sequence with positive hybridization to the probe (Fig. 3). A single positive band of 0.83 kb was identified in one of the three plasmid constructs and this construct was used in all further studies, including DNA sequencing.

**DNA sequence analysis**

Direct plasmid sequencing is an alternative to the use of single-strand M13 vectors and obviates the need for DNA subcloning or the production of a detailed restriction map. In order to evaluate the plasmid construct which gave a positive hybridization signal against the enterotoxin probe, we chose to sequence directly from the plasmid without further characterization. As a first step, a single 17 bp primer sequence complementary to the deduced
sequence for amino acids 9 through 14 of the toxin gene was synthesized. An arbitrary base was chosen for those positions in the sequence exhibiting third-base degeneracy. Using this primer, CsCl-purified plasmid DNA was sequenced using reverse transcriptase, as indicated in Methods. Despite the fact that this primer was subsequently found to be incorrect in 2 out of 17 bases, a readable though limited DNA sequence was obtained which allowed the unambiguous assignment of upstream sequences for the synthesis of a new sequencing primer. A clear correlation was obtained between the DNA sequence obtained in these studies and the deduced codon assignment for the *C. perfringens* enterotoxin gene. Fig. 4 shows the DNA sequence from a Klenow reaction reading into the toxin gene from a 21 bp primer hybridizing with the DNA codon sequence for amino acids 26 through 32 (DNA coordinates +76 to +91). By using sequence-deduced primers, both strands of the N-terminal and upstream region of the toxin gene have been determined (Fig. 5). Comparable results were obtained with both Klenow- and reverse-transcriptase-catalysed sequencing reactions. However, we have found it helpful to utilize both procedures as, on occasion, both enzymes can encounter DNA sequences which cause premature termination. In our experience such DNA regions are unique for one or other enzyme and these enzymes are, therefore, complementary at overcoming sequencing artefacts.
The DNA sequence 5' to the toxin structural gene

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Fig. 5 shows the DNA codon sequence for the first 30 amino acids of the C. perfringens enterotoxin gene accompanied by 300 bp 5' to the methionine initiation codon. These first 30 amino acid codons correlate precisely with the previously published amino acid sequence for the toxin protein (Granum, 1985). The 300 bp sequence 5' to the translation start has an 83% AT base composition and contains some interesting features. A Shine–Dalgarno consensus (AGGAGATG) (Shine & Dalgarno, 1975) can be identified with 11 bases separating this from the initiation ATG. This compares with an identical sequence found 14 nucleotides upstream of the C. tetani tetanus toxin sequence (Eisel et al., 1986).

A region with significant homology to the putative −35 and −10 promoter sequences of both the C. tetani tetanus toxin gene (Eisel et al., 1986) and the Bacillus cereus penicillinase gene (Sloma & Gross, 1983) is located 5' to the Shine–Dalgarno sequence (Fig. 5). The homologous sequences are at −265 bp and −239 bp with respect to the enterotoxin initiation codon and represent a putative promoter sequence for the toxin gene. However, there are other regions of homology with these sequences from C. tetani, for example bases −97 to −86 inclusive are identical to the −35 sequence. A definitive identification of the transcription initiation site awaits additional experimental data.

The availability of a DNA clone should facilitate a detailed structural and functional characterization of the enterotoxin. In particular, a putative precursor–product relationship for the toxin can be resolved (Smith & McDonel, 1980) and N-terminal fusions of the toxin gene promoter to expression markers such as β-galactosidase or bacterial luciferase will allow an analysis of the induction of gene expression during sporulation. Finally, hybridization to C. perfringens chromosomal DNA, isolated from both toxigenic and non-toxigenic strains, may shed new light on the nature of toxin production from food poisoning isolates.

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REFERENCES


