A complex array of Hpr consensus DNA recognition sequences proximal to the enterotoxin gene in *Clostridium perfringens* type A

Sigrid Brynestad,¹ Lesley A. Iwanejko,² Gordon S. A. B. Stewart³ and Per Einar Granum¹

Author for correspondence: Per Einar Granum. Tel: +47 22 96 48 45. Fax: +47 22 96 48 50.

¹ Department of Pharmacology, Microbiology and Food Hygiene, Norwegian College of Veterinary Medicine, PO Box 8146, Dep., N-0033 Oslo, Norway

² Department of Genetics and Microbiology, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

³ Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington Campus, Leicestershire LE12 5RD, UK

Enterotoxin production in *Clostridium perfringens* is both strain dependent and sporulation associated. Underlying these phenotypic observations must lie a genetic and molecular explanation and the principal keys will be held within the DNA sequence both upstream and downstream of the structural gene *cpe*. In accordance with the above we have sequenced 4.1 kbp of DNA upstream of *cpe* in the type strain NCTC 8239. A region of DNA extending up to 1.5 kb 5' to *cpe* is conserved in all enterotoxin-positive strains. This region contains a putative ORF with substantial homology to an ORF in the *Salmonella typhimurium* IS200 insertion element and, in addition, contains multiple perfect consensus DNA-binding sequences for the *Bacillus subtilis* transition state regulator Hpr. The detailed structural elements revealed by the sequence analysis are presented and used to develop a new perspective on the molecular basis of enterotoxin production in this important food-poisoning bacterium.

Keywords: *Clostridium perfringens*, enterotoxin, Hpr, IS200, *cpe*

INTRODUCTION

Clostridium perfringens type A is responsible for the classic mild type of food poisoning. Illness is caused by an enterotoxin produced in the small intestine by sporulating bacteria with release of the toxin during mother cell lysis (for reviews, see Granum, 1990; Granum & Stewart, 1993). Enterotoxin production may vary between strains by as much as three orders of magnitude (Skjelkvåle *et al.*, 1979; Granum *et al.*, 1984), at least in laboratory media, and inclusion bodies containing the enterotoxin are sometimes observed within the cells (Skjelkvåle & Duncan, 1975). The use of sporulation mutants has established that enterotoxin is synthesized prior to stage III of sporulation (Duncan *et al.*, 1972), and can be detected immunologically in sporulating cultures after 2–3 h, corresponding to stage II (Duncan, 1973). Stable enterotoxin mRNA with a half-life of some 58 min has been isolated (Labbe & Duncan, 1977), but any possible

heterogeneity of transcripts has not been investigated. The enterotoxin has been cloned in a number of laboratories (Van Damme-Jongsten *et al.*, 1989; Iwanejko *et al.*, 1989; Hanna *et al.*, 1989), and with the development of a restriction map of the *C. perfringens* genome (Canard *et al.*, 1992) there is now an opportunity to map the *cpe* gene in relation to other genetic markers on the chromosome.

The ability to produce enterotoxin is not associated with all *C. perfringens* type A strains; indeed only about 6% of food-isolated strains exhibit this capacity (Van Damme-Jongsten *et al.*, 1989). It also seems that the ability to produce enterotoxin can come and go within individual culture isolates. Despite past speculation that this relates to changes in gene regulation or even transposition, the underlying genetic basis for these phenotypic observations has not yet been established. As part of the cloning strategy for *cpe* (Iwanejko *et al.*, 1989) we have previously isolated a substantial region of the DNA 5' to the structural gene. This DNA should hold many of the keys to the unusual expression phenotype for enterotoxin, as within this region we would expect to find the regulatory elements that specify both an association with sporulation

The EMBL/GenBank/DBJ accession number for the sequence reported in this paper is X71844.

and strain variation. In this paper we present the sequence of 4.1 kbp of DNA 5' to *cpe*. We have compared this region across several strains of *C. perfringens* and we propose a link between an Hpr-like transition state regulator and the sporulation-associated expression of *cpe*.

METHODS

Strains. All the *C. perfringens* strains (see Table 3) were from the culture collection of the Norwegian Food Research Institute, Ås, Norway. The type A strains are all known to be enterotoxin positive (Skjelkvåle *et al.*, 1979), while the type C has been found to be negative (Skjelkvåle & Duncan, 1975).

Isolation of chromosomal DNA. Chromosomal DNA was extracted from the *C. perfringens* strains using the method of Pitcher *et al.* (1989). Cells were grown overnight in 100 ml de-aired BHI medium (Oxoid) within an anaerobe flask at 37 °C. Cells were harvested by centrifugation (10000 *g* for 15 min at 4 °C). The pellet was resuspended in TE buffer with 500 µl lysozyme (50 mg ml⁻¹) and incubated at 37 °C for 30 min. Then 2.5 ml GES solution was added (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% sarcosine). After lysis (rapid) the samples were placed on ice for 10 min; 1.25 ml 7.5 M ammonium acetate was added, and the samples were again placed on ice for 10 min. The samples were then extracted with 2.5 ml chloroform/isoamyl alcohol (24:1, v/v), mixed and centrifuged (19000 *g* for 15 min). The supernatant was carefully pipetted out and 0.54 vol. cold 2-propanol was added and mixed, and then centrifuged (4000 *g* for about 1 min). The pellet was washed with 70% (v/v) ethanol, dried, and dissolved in 200 µl distilled water.

Plasmid isolation. *C. perfringens* plasmid DNA was isolated either by the use of Magic Miniprep kit (Promega, USA), following the manufacturer's instructions after 1 h preincubation of the bacterial pellet with 250 µg lysozyme (Sigma) plus 10 U mutanolysin (Sigma), or by the following method. Cells were grown in 50 ml BHI containing 0.4% glycine within an anaerobe flask until late exponential phase, then centrifuged (3000 *g* for 15 min) and resuspended in 1 ml Tris-sucrose buffer (50 M Tris/HCl pH 8.0, 5 mM EDTA, 50 mM NaCl in 25% sucrose). Lysozyme (0.4 ml of 50 mg ml⁻¹) and mutanolysin (0.1 ml of 2000 U ml⁻¹) were added and the samples incubated at 37 °C for 15 min. EDTA was added up to 60 mM, and the samples were incubated at 37 °C for 10 min. The sample was cooled on ice and 10% SDS was added to 1% and left on ice until clear (5–10 min) NaCl was added to 0.75 M, and the samples were left on ice for 20 min, then centrifuged (19000 *g* for 20 min). The DNA was precipitated with 2 vols ethanol at -20 °C overnight. The pellet was dried under vacuum and dissolved in 200 µl TE.

Plasmids from *Escherichia coli* were isolated either as described in Sambrook *et al.* (1989) or using Magic Minipreps (Promega) according to the manufacturer's instructions. All sequenced DNA was prepared using the Magic Miniprep kit.

DNA hybridization. Samples were fractionated on gels, which were dried in a vacuum system (Bio-Rad). The gel was denatured (and deprobed) in 0.5 M NaOH, 0.15 M NaCl for 20 min, and neutralized in 0.5 M Tris/HCl pH 7.5, 0.15 M NaCl for 20 min. The gels were hybridized in a solution containing 6×SSC (1×SSC contains 0.15 M NaCl and 0.015 M sodium citrate), 5×Denhardt solution (1×Denhardt contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin fraction V) and 0.05% sodium pyrophosphate in a sealed plastic

bag. Probes labelled with ³²P were added (25 ng multiprime-labelled DNA, 200 ng end-labelled) and hybridized for 18 h at temperatures from 40 °C to 48 °C depending on the desired stringency. The gels were given two or three 20 min washes in 6×SSC, 0.05% sodium pyrophosphate at temperatures in the same range as hybridization. Gels were wrapped in Gladpack and exposed to Amersham hyperfilm with an intensifying screen at -70 °C. Transformations were performed using competent *E. coli* strains JM105 or XL-blue as described by Hanahan (1983).

DNA sequencing. Plasmids were sequenced using T7 DNA polymerase and a fluorescent primer (Kristensen *et al.*, 1988). Standard universal and reverse primers for pUC18/19 vectors were used. The products were analysed and the sequence derived using an instrument constructed at EMBL, Heidelberg, Germany (Ansorge *et al.*, 1986, 1987), at the Biotechnology Centre of Oslo, sequencing laboratory.

Polymerase chain reaction. PCR reactions were carried out in a Perkin Elmer Cetus DNA Thermal Cycler using the following programme: 30 cycles at 92 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The GeneAmp PCR Core Reagent kit (part no. N808-0009, Perkin Elmer Cetus) was used according to the manufacturer's instructions.

RESULTS

Sequence analysis of DNA 5' to the *cpe* gene

The *cpe* clone pLW1 (Iwanejko *et al.*, 1989), which was used in the identification and sequencing of *cpe*, contains a 6.8 kb DNA insert in plasmid pHG165 (Stewart *et al.*, 1986). *cpe* and 300 bp 5' to the gene were previously sequenced, and regions with homology to a Shine-Dalgarno consensus sequence and the -35 and -10 promoter sequence for the *Clostridium tetani* toxin gene identified. This initial sequence strategy did not require detailed restriction analysis of the 6.8 kbp clone and, in consequence, this present study commenced with the development of a more detailed restriction map (Fig. 1), subsequently used for subcloning the DNA into pUC18 plasmids (Yanisch-Perron *et al.*, 1985) for further sequencing. Overlapping clones were obtained over a 2 kbp area and nested deletion clones, using a Pharmacia kit, were made for the remaining areas with no suitable

Fig. 1. Restriction map of the *cpe* clone pLW1 (Iwanejko *et al.*, 1989). The restriction sites are indicated as BglII (Bg), ClaI (C), HindIII (H), KpnI (K), NcoI (N), SstI (Ss), StuI (St) and XbaI (X). The polylinker of the cloning vector, pHG165, is indicated as poly. Three different probes used are indicated: a 458 bp HindIII fragment, and a K-Bg and a Bg-Bg (poly) fragment in the *cpe* gene. The dotted area indicates the *cpe* ORF; the shaded area 5' to *cpe* has been sequenced before. The open area indicates the new sequence presented in this paper.

Fig. 2. Map of sequenced DNA from pLW1. Top: indication of open reading frames (ORF), direct repeats (r), palindromes (p) and sequences homologous to the recombination sites of Tn3. The position of the primers from Table 3 used in the PCR studies are indicated as arrows on the top of the map. Bottom: arrows indicate the different subclones used for sequencing. The restriction sites are as in Fig. 1.

restriction enzyme sites. Plasmids were subjected to automated sequencing protocols and the final sequence of 5090 bp was constructed with the use of programs from the GCG package (Genetics Computer Group, 1991). The enterotoxin gene and some 3 kbp of proximal DNA has either been sequenced in both directions or has been sequenced several times from separate clones (Fig. 2). The initial 1.7 kb of the 6.8 kbp clone (the region most distant from *cpe*) gave consistently poor sequencing results in the attempts made, and is not included here. The 5090 bp sequenced starts at a *Kpn*I site, and this is considered bp 1 (Fig. 3). Using this convention the *cpe* gene coding area starts at bp 4131. There is a gap of a few bases after bp 1150 which repeated attempts at sequencing have failed to resolve.

Possible open reading frames (ORFs) were found using the GCG package. In addition to *cpe*, three ORFs were found: ORF1 from bp 369 to 930 (187 amino acids), ORF2 from bp 2134 to 1375 (253 amino acids) and ORF3 from bp 2653 to 2920 (89 amino acids). In addition to the above there may be an ORF running into the 5' end of the sequenced region as bps 0–399 have an ORF overlapping ORF1. All the sequences and the amino acid translations were compared against known sequences using the FASTA, TFASTA and BLAST programs (Pearson & Lipman, 1988; Altschul *et al.*, 1990). Only one significant homologous DNA and one protein sequence was found, and only for ORF3 (Fig. 4). This search produced one striking homology at both the DNA and protein level. ORF3, which is the nearest ORF 5' to *cpe*, shows 66% DNA homology and 63% amino acid homology to a putative ORF in the *Salmonella typhimurium* insertion sequence IS200 (Fig. 4; Gibert *et al.*, 1991). Ribosome-binding sites (SD) and putative –35 and –10 regions can be located 5' to all four ORFs (Table 1). Only one plausible SD and –35 and –10 promoter sequences could be detected 5' to three ORFs upstream of *cpe*. In contrast, at least ten

possible –35 and –10 promoter sequences can be identified upstream of *cpe*. Nine of these –35 sequences are partly homologous to other known –35 regions from clostridia (Sebald, 1993).

Searches for repeats and palindromes were performed. There was a marked concentration of repeats within 100 bp of each other in the DNA separating the probable ORFs. Between ORF 1 and ORF 2 there are four perfect repeats, five repeats were identified between ORF 3 and *cpe*, and four palindromes were located between ORF2 and ORF3 (Figs 2 and 3). Sequences resembling the Tn3 recombination site (Sherratt, 1989) were found in multiple copies 5' to *cpe*, and one copy was found 3' (Table 2). The two closest to the *cpe* gene are marked on Fig. 2.

A consensus DNA recognition sequence for the *Bacillus subtilis* DNA binding protein Hpr has been deduced from the examination of 10 separate DNase-1-protected regions on four separate genes (Kallio *et al.*, 1991; Strauch & Hoch, 1993). This sequence is AATANTATT, with the 5' half being more highly conserved than the 3' half. Since Hpr has been implicated in the control of many transition state functions in bacilli, a similar system may operate in clostridia. Given the known association with sporulation for *cpe* expression, regulation by an Hpr homologue would be consistent with current knowledge. The sequence between ORF3 and *cpe* contains three pairs of perfect Hpr protein consensus binding sites and, in addition, there is another perfect consensus 3' to *cpe* (Fig. 5). This result is important since it is known that both upstream and downstream Hpr binding sites are required for the full repressive effect of the transition state regulator (Strauch & Hoch, 1993). Using oligonucleotide primers homologous to *hpr* (sequences 119–139 forward and 685–704 reverse primer) (Perego & Hoch, 1988) we isolated from agarose gels, a 586 bp fragment from *hpr* by PCR. Using the primers for PCR on clostridial DNA was not successful, but Southern hybridization with a ³²P-

1 GCTCGAGTAC CCGTAGTTGC ATTAATAGGA TTAACCTTAC TTCTGTATC
51 AATCGATTGG GCCGCGAGGTG GTGCTGGTTC AGCTAATTAT GCTAGTTTAG
101 AAAATTTAGC TGTGGCTATG TTGTATTAA TAATAACTCT TTTACTTAAT
151 AACTATGGAA AAGGAATGAT AAGTAGTGCT TCAATACTTA TAGGTATAGT
201 TGTAGGATAC ATAGTTTGTA TTCCACTTGG ACTAGTTGAC TTTACTTCTG
251 TAAAGGAAGC AAGTTGGCTA TCATTTCCTA AGATCCTTGA ATTCGGAGTA
301 ACTTTTGATG CTAAGGCAGT TATGGCCTTC ATACCTGCTT ACTTTGTAGC
351 CACAATAGGA ACTGTAGGAT GCTTAAAGC TATTGGAGAG ACATCTTAAT
401 ATTGATATTT GGTGATAAAA GAGTGGCCGC TGGAGTATTA TCTGATGGTG
451 TAGGAAGTGC ATTAGGTGGA CTAGTAGGTT CTTGCTCTAA TACTTCTTTT
501 AGTCAAAATA TAGGTATTAT TTCTCTAACT AAGGTAGCTA GCCGTCATGT
551 AGCTGTAATG GCTGTATATC TATTAGTTAT CTTGGGCTTC TTACCABAAG
601 TAGCCGCTAT AATAACAGGA ATACCAAAAC CAGTTTTAGG TGGCGTAGGT
651 ATAATGATGT TTGGAACAGT TGGGCGAGCT GGTATTAGAA CACTAAGTAA
701 TATAAACTT ACAGAGCGTA ATCTTTTAAT AATAGCTATA TCTATGGGGC
751 TTGGACTAGG AGTTACATTT AGACCTGATG TAATTCATAA TCTTCCAGAA
801 GCAATTAGAA TGATCTTCTC TTCTGGAATC TCAACTGGAA CAATAGCTGC
851 CCTAATCCTA AATGCAGTAT TAAAGAAAG TCCAACATCA ATGGAATTTG
901 AAAATATGTA TGATGAAGAA ACAAGGCTA GTTAAAAAGT TTAAAGTTA
951 ATAAATATAA TAATCTTAAC TATTCACATAT TAATAAATAA AATCTAATTA
1001 AAAACTAAAT ATATTTTAT TACATAATAT ACAAACTAA ATTGCAATAT
1051 CAAATTGAAC TAGTCCTTAA TTATGATTTA AGAACTAATT AATATAATGA
1101 ATATATTTTA TCTAAGTGTA TTCAAAATA CTCTTCTGGA GTCTGTAAAT
1151.. CTCTAGGA AGAGATTACA TCCAATTTTC TATGAAAGAT ATAGTTTCAA
1201 GTGAGTAAAT TGAATTTCTG TTCCCTTTCG GGATAAAACG CCTAATCAGT
1251 CCATTATGAC GCTCATTTGT ACCTTTTTC AATGAAGAAT ATGGGTGAGT
1301 GAAATACACT TTTAGTTTTT TGTTTTTGT TCAAACTCTG ATAAATCAGC
1351 AATTCAGAGC CATTATCAGC AGTTATCTC TTAATACTT CACTAATTTT
1401 ACTGCCCAA ATTCTTTAA TCTTATCAAG AGCCTTTGTA ACAGATATTG
1451 TTGAATGGGA AGACATTTCA GATATTATAG CATACCTAGT TTTACGCTCG
1501 ACAAGTGTTA ACAAAACCTT ATCTTTATTA GATTTTTCAC CTAATACACA
1551 GTCTATTTCG CAATGTCCAA ATTCTTCAGC GTTTTCAATA GAATTAGGAC
1601 GATCTGAAT GCTAGTGCCA AGCTTTTCT TATTATTCT TACTCTAGTA
1651 GACTTTTAAT TTCTATGAAG TTTAGCTGCT AAATCTATAT TTTTATAGG
1701 TAATAAGCCA AGGTCTACAT AGTTATBAAG CGTTTTGTT GAAATAATTT
1751 GAGAAGGACT AAATCTAGAT GAATGCAGAG CTTACCAAC ACATGCATCT
1801 AAAGACCAAT GATCATCTT AACTTTATCA ACTACATATT TTATAAATC
1851 ACTACATCTT AATAGTTTGT ATTTACGATT AGATTTTAA CGATTCTTCT
1901 TATAACAGC TTCACCAGTA TCAGCAAAAT ACACATGAAA TTCCCTAACCT
1951 TGCTTAATTT GCTTAGTAGT ACCACGGCTT ATTTGCTTTA AAACGGTATT
2001 TATAGGCCTA TTTAGTTCTT TTGTATTTTA TAAGCTGAAA ATCCATCTTT
2051 AAGACGTATT TCAACTATCA TAGGCTCTTT CATATTTAAG TGTATTATTT
2101 TTCTTGACTC TGTTTATGA TTTTGATAGT CCAATGGTAT TATCCTCCGT
2151 GTGTTTTATT TTTTACGCG ATTAATCAT AACACAGGT AATCCACCAT
2201 GGATTTTTT ATTTAGTTCA ATTTCAATTT ACAATTAAGC GTATAATAAT
2251 TTTAAGCTAT TACTATATGT GAATAGTCTT TCCTTTATTT GAAATTAAGA
2301 AAACAAGAA AGCTCTATAA AATAAGTAGG GCTTCTTGT TGIGTAATGA
2351 AAACCACTTG CTATCGAGT GTGTTCAGAA GAGCTTTAGC GATTGTAAGA
2401 AAAAACCTCC TTTTGTAAA ATGAAAATAG GTTCGTAGCC AATTTTCGTA
2451 ACAAAAGGAG GGTTTTAAAT GGATACAAAC AGTTTAGCAC ATACAAAATG
2501 GAATTGTAAA TATCATATAG TTTTGCACC AAAACACAGA AGGAAGAGAA
2551 TATATGGAGA GAAAAACAA GAAATAAGTG AGATATTAA GCAGCTTTGT
2601 GAATGGAAAG GAGTTAGATA GTCGAAGCGC ACGCTTGCCT AGATCACATA
2651 CACATGCTTG TTGAAATACC ACCTAAAATG AGTGTTCCTA GTTTCGTTTG
2701 GGTCTAAAA TGTAAAAGTA GTCTTATGAT TTTTGAGAAA TTGCAAAAT
2751 TAAAAATATA ATATGGAAAT AGACATTTCT GGTGTATGGG ATTTTACGTT
2801 GATACTGTGC GAAAAATATA GAAGGCAATT GAAGACTATA TAAGAAATCA
2851 AGARCAAGAA GATATGATAG CTGATCAAAAT AAGTTAAAA GAATATATGG
2901 ACACTTTAA GTGTAGCAAG TAAAGAAGTA TCGCGCTGCG GGACTAATTT
2951 AAGCGTCTTA AGATGCAACG TAGGTAGTAT GCCCTTATAG GGCTAAGGAA
3001 ATACCACCAG CTAAGCTGCT GGATTTTTAT TTTAACTTAG GCACTTATAC
3051 ATAAAGTCTT TTAATATTTT TAACCTAAAT TTCTATAAAT GTTAATTTGT
3101 AAATTATTAC TAAGATAAAT ATTAATAGTG TATACATTGA GATAGTAAAT
3151 AGCATGATAT TTAATTTTTT AAAGTAATTT TAATAAATTT ATATAATTTCT
3201 GAAAAATATA AAGGTAGCTC ATTTTAGAGC TACCCATAAT ACGATTAGAA
3251 ATCTTTTTAT TTCTATTTTA TTATTAGTTA AAAATCTATA ATTTTATTTT
3301 ATACATATTT TCTACTTTTC ACAATACTG ATGAAATAGA AACTCTAAAT
3351 GAATCAGATA CATCACATTA ATGTTCCACA ATTCATTTTG TATACAATCT
3401 ATTAACCTGA AGAATGGATT AGTTTATCAA AACTTTAAT AGAGTTATTA
3451 TTATATTTAT TAAAACTCT CTCCTTTAAA TTAAGTTGAT ATATATGAAA
3501 AATACATACG CAGGGTTAGA GATAAAAAA GTATTCAGT TTATAAAAAA
3551 CTTAGAGTAT CTATAAACTT GATACTCATT TATTAATAAG ATAAATTTAT
3601 TTATTTTTAT CTTATTAATC GCTATATTCA TTATAGTTAC TTGTTGGGGT
3651 ATTTATTACT CCAATAAGTG CCAAAATTTA GAGTATAGCT TTAACATTTT
3701 CTTGATAGTT ATCTGGTAAA ATATTAAAT TAAATCCTTG TAAACCAAG
3751 GAAATTAAGG TTGCTATAGA AAGCCATAAC CCATAATTTCT TAAACGCTGA
3801 TTTATTTATT ATAATATATC ACTCTCCTTG TTTATAATAT ATAATATTAT
3851 GTTTAGTGAA ATTATGTTAA TATACTACTT ATTTCTCTCT TTATATTAAT
3901 TAACATTICA ACTTGAATCT TTAACGTAT ATCTCTTTTA TTACCCAAGC
3951 TTTAATCTCT TCAGCAATTA TATCATAAAA TGCCAATGA GAAATATATT
4001 CAAGATTATT AAAGATATAT ATTATAATTT AATATTTTGT TAATACTTTA
4051 AGGATATGTA TCCAAAATAA AAACCTTTTA ATAATATATT ATATAAAAAA
4101 AATTAGAAAT AAGGAGATGT TAATTATAAT ATGCTTAGTA ACAATTTAAA
4151 TCCATGGGTG TTGAAAATG CTAAAGAGT ATTTCTTATT TCTGAGGATT
4201 TAAAAACACC AATTAATATT ACAACTCTTA ACTCAATTT AAGTATGGA
4251 TTATATGTAA TAGATAAAGG AGATGGTTGG ATATTAGGGG AACCCCTCAGT
4301 AGTTTCAAGT CABATCTTA ATCTAATGA AACAGGTACC TTTAGCCAAAT
4351 CATTAACTAA ATCTAAGAAA GTATCTATAA ATGTAATTTT TTGACTTGGA
4401 TTTACTTCTG AATTTATACA AGCATCTGTA GAATATGGAT TTGGAATAAC
4451 TATAGGAGAA CAAATACAA TAGAAAGATC TGTATCTACA ACTGCTGGTC
4501 CAAATGAATA TGTATATTAT AAGGTTTATG CAACCTATAG AAAGTATCAA
4551 GCTATTAGAA TTCTCATGG TAATATCTCT GATGATGGAT CAATTATATA
4601 ATTAACAGGA ATATGGCTTA GTAAACATC TGCAGATAGC TTAGGAATAA
4651 TGTATCAAGG TTCATTAATT GAACTGGTG AAAGATGTGT TTTAACAGTT
4701 CCATCTACAG ATATAGAAAA GGAATCTCTT GATTTAGCTG CTGCTACAGA
4751 AAGATTAAAT TTAAGTATG CATTAAACTC AAACCCAGCT GGAAATTTAT
4801 ATGATTGGCG TTCTTCTAAC TCATACCTTT GGACTCAAAA GCTTAATTTA
4851 CACTTAACAA TTACAGCTAC TGAACAAAAA TATAGAACTT TAGCTAGCAA
4901 AATTGTTGAT TTTAATATTT ATTCABAATA TTTTAATAAT CTAGTGAAT
4951 TAGAACAGTC CTTAGGTGAT GGAGTAAAAG ATCATATGTT TGATATAAGC
5001 TTAGATGCGG GACAATATGT TCTGTAAAG AAAGCTAATT CATCATATAG
5051 TGGAAACTCT CACCCCTATT CAATATTATT TCAAAAATTT

Fig. 3. For legend see opposite page.

labelled 586 bp *B. subtilis* DNA fragment gave a positive hybridization band for all the *C. perfringens* strains (Table 3). We are currently seeking a gene with homology to *hpr* from *C. perfringens*.

Hybridization studies on DNA from various *C. perfringens* strains

Chromosomal DNA was isolated from nine *C. perfringens* strains (Table 3) which were tested for toxin production with a latex agglutination test kit (Oxoid). A 240 bp *KpnI*-*BglII* probe for the *cpe* gene, and a 458 bp *HindIII* fragment upstream from *cpe* (Fig. 1) were labelled with ^{32}P and used in Southern hybridization with *EcoRI*-digested chromosomal DNA separated on a 0.7% agarose gel. The *cpe* probe gave a single signal in the enterotoxin-positive strains, and no signal in enterotoxin-negative strains (Table 3). Despite the above, the hybridization band position was different for each strain, suggesting significant heterogeneity in chromosomal location.

The 458 bp *HindIII* fragment (determined to be 330 bp on agarose gel: Granum & Stewart, 1993) gave a very different picture, with hybridization band numbers varying from one to three to multiple. These results extended the previous studies with *C. perfringens* NCTC 8239 (Granum & Stewart, 1993), where multiple repeats of this region were first identified. Two of the enterotoxin-negative strains gave positive hybridization with this probe, including a type D strain; of particular interest, however, was that one of the enterotoxin-positive strains gave no hybridization signal, indicating that this region can be uncoupled from enterotoxin expression.

PCR studies of DNA upstream of *cpe*

The analysis using the two probes described above indicated that a region 2.5 kbp upstream of *cpe* may be unlinked to the regulation of enterotoxin production. However, the potential importance of ORF3 and the *Hpr* consensus binding sequences remain unclear and an extension of the above study to incorporate these areas in a linking study should prove helpful. A PCR approach was used to indicate the presence in diverse *C. perfringens* strains of the sequences up to 4 kbp 5' to *cpe* in *C. perfringens* NCTC 8239. PCR primers were constructed according to Table 4 and Fig. 2 and these provided amplification of three separate but partly overlapping regions proximal to *cpe*. Of the six enterotoxin-positive strains tested only two in addition to NCTC 8239 gave PCR fragments of the expected size, indicating an equivalent genetic structure (Table 3). The PCR1 1213 bp fragment and the PCR2 1697 fragment were either absent

or truncated in all of the remaining strains, indicating, as above, that these regions are unlinked to *cpe* expression. Of particular significance, however, was the finding that the PCR3 1397 bp fragment, which includes most of ORF3 and all of the intervening region up to *cpe*, was present in the correct size in all enterotoxin-positive strains.

DISCUSSION

About 4.1 kb DNA upstream of the enterotoxin gene from *C. perfringens* NCTC 8239 has been sequenced and subjected to detailed analysis. We have identified three ORFs, with possible associated control sequences, upstream of *cpe* (Fig. 2). ORF1 and ORF3 are found on the same coding strand as *cpe*, while ORF2 is on the other strand. Of the region associated with *cpe* in strain NCTC 8239 only that from the start of ORF3 to *cpe* is conserved among enterotoxin-positive *C. perfringens* strains. It is reasonable to assume therefore that ORFs 1 and 2 and their associated intergenic sequences are not an integral part of the control of *cpe* expression. Nevertheless the potential involvement of these regions in mediating the overall level of *cpe* expression cannot be excluded. Given that ORF3 does appear linked to *cpe* in all strains it is particularly interesting that it has such high homology with the C-terminal end of a putative ORF from *S. typhimurium* insertion sequence IS200 (Fig. 4). It is possible that the IS200 sequence actually produces this peptide as homology starts with ORF3 methionine and shows 93% conservation over the first 14 amino acids. At present the functional significance of this is unknown, but two additional observations support the association of the ORF3 region with mobilizable elements. Firstly the DNA sequence which includes ORF3 is completely devoid of 6 base restriction endonuclease palindromes. Such an absence is strongly suggestive of selection pressure derived from interspecies DNA transfer which might occur on mobilizable plasmids or bacteriophages. The absence of 6 base palindromes in T7 for example has been ascribed to elimination by phage replication in multiple hosts having diverse type II endonucleases (Rosenberg *et al.*, 1979). In this regard it is also interesting to note that *C. perfringens* enterotoxin, like the *Staphylococcus aureus* enterotoxins, is a superantigen (Bowness *et al.*, 1992). The term superantigen has been applied to a group of bacterial and retroviral proteins that stimulate a large number of T lymphocytes in a manner distinct from that of classic antigens. Since at least some, if not all, of the *Staph. aureus* enterotoxins are known to be phage mediated (Alouf *et al.*, 1991) it is quite possible that the superantigenic enterotoxins are of viral (phage) origin. Returning to the second factor indicating the potential for *cpe* mobilization, there are sequences resembling the *Tn3* recombination site both upstream and downstream of *cpe* (Table 2). It

Fig. 3. The sequence of 5090 bases from the *KpnI* site (Fig. 1) 5' to *cpe*. The sequence of the *KpnI*-*KpnI* fragment (Fig. 2) is from this study, while the sequence of the remaining part of *cpe* and 99 bases 3' of the gene is from Iwanejko *et al.* (1989) and Van Damme-Jongsten *et al.* (1989). The four ORFs are underlined.

ORF3	228	TATTGATCAGCTATCATATCTTCTTGTCTTATTCTTATATAGTCTTCAATTGCCTT	169
IS200	150	TAATTGCTCACCATTATCTCTTCAAGCTGGTCTTATGATGCTGTATCTCGG	209
ORF3	168	CTTATTTTTCGACAGTATCAACGTAATAATCCATACACAGAAATGCTATTCCATA	109
IS200	210	CGTCTTCTTACCCACCGTATCGACATAGTACCTCTGACACAGAACTCCCTGTTCTGTA	269
ORF3	108	TTTATATTTTAAATTTGCAAAATTTCTCAAAAATCATAAGACTACTTTTACATTTTGAAC	49
IS200	270	TTTGAATTTTAGATCCCAAACTGCTCGTAAAGCATCAGACTACTTTTACCCCTCAGATA	329
ORF3	48	CCAAACGAACTTGAACACTCATTTTAGGTGGTATTCAACAAGCAT	1
IS200	330	TCCCATGAACTCGACACACTCATCTTCGGCGGATCTCCAGAAGCAT	377

		10	20	30
ORF3		MLVEIPKMSVSSFWVWLKCKSSLMIFEKF		
IS200	VGQXAAYXENCNGKTYEFLEAECCADHIMLLEIPKMSVSSFMGLKGSMLMIEQF			
	90	100	110	120
	130	140		

	40	50	60	70	80
ORF3	ANLKYKYNRHFVCMGFYVDTVGKKNKAIEDYIRNQEQEDMIADQISLKEYMDTFKCSK				
IS200	GDLPKYRNRREFWCRGYVDTVGKNTAKIQDYIKHQLLEEDKMGQLSIPYPGSPIYGPXV				
	150	160	170	180	190
	200				

Fig. 4. Sequence homologies between the DNA and translated amino acids of ORF3 and the IS200 from *S. typhimurium* (Gibert *et al.*, 1991).

Table 1. Putative ribosome-binding sites (SD) and promoter sequences found 5' to the ORFs in Fig. 3

The relative positions of the sequences to the start codon of the ORFs are shown.

	SD sequence	Promoter sequences	
		−35 region	−10 region
ORF 1	−5	−195	−170
	AGGAACTG	AATGATAAG	TATA
ORF 2	−50	−132	−107
	TTAACCTG	TATTCACATT	TTATA
ORF 3	−37	−159	−134
	AGGAGTTA	GTTTAGCACATAC	TATA
CPE	−11	−28	−3
	AGGAGATG	ATATAAAAAAAT	TTATA
		−60	−35
		TCCAAAATAA	TATA
		−157	−132
		TTCAGCATTAATAT	TATA
		−260	−235
		AAATTATGTTAA	TTATA
		−281	−256
		ATATAATATTA	TATA
		−520	−495
		ATTTATTTTAT	TTATA
	−590	−565	
	ATAGTATTCAAGT	TATA	
	−946	−921	
	TAATTTTAAT	TATA	
	−961	−936	
	ATATTTAAATTTT	TATA	
	−1021	−996	
	TGAAATTATTA	TATA	

Table 2. Tn3 recombination site consensus comparison

		Position relative to <i>cpe</i>
5'	AATATTTAACTT	−1055
	TATTATTAGTTA	−850
	AGTTATTATTATA	−576
	AAATTATTTA	−528
	AAATATTAATTTT	−399
	AATATATAATATTAT	−280
	AAGATTATTAA	−118
	AAATAATATATTATATA	−36
3'	AATATTATTTCAAAAA	+80
Tn3*	AAATATTATAAATTAT	

* Sherratt (1989).

Fig. 5. The positions of the Hpr binding sequences up- and downstream of the *cpe* (the numbering of the bases at the top is as in Fig. 3). The sequences of three pairs of Hpr binding sites upstream to *cpe* are: AATAGTATT, AAAATTATT; AATATTAAT, AATATTATG; AAGATTATT, AATAATATA (starting point of each of the sequences: 3527, 3593, 3720, 3843, 4002 and 4080). A single Hpr binding sequence is observed downstream of *cpe*: AATATTATT (starting point 5072).

certainly appears that excision of *cpe* and the DNA region including ORF3 is possible since *C. perfringens* 2154 which has previously been described as enterotoxin positive (Skjelkvåle *et al.*, 1979) now appears deleted in this region (Table 3).

It has long been known that some strains of *C. perfringens* produce enormous amounts of enterotoxin, and the possibility of multiple copies of *cpe* scattered around the genome has been a plausible explanation. This theory can however be ruled out, as only a single *cpe* gene is found in such strains, located at the position close to the origin of genome replication (Canard *et al.*, 1992). Since mRNA stability appears to be excluded (Labbe & Duncan, 1977), a possible explanation could be that differential use of at least some of the 10 putative RNA polymerase binding sequences upstream of *cpe* allows for different levels of transcription and/or coupled translation. This aspect is currently being examined by isolation of mRNA during sporulation of *C. perfringens* and transcript mapping.

For the first time since the early observation of Duncan *et al.* (1972) a clear molecular link between enterotoxin production and sporulation has been discovered. An Hpr-

Table 3. *C. perfringens* strains used for hybridization and PCR studies

	Type	Probes*			PCR primers used*		
		Hpr	<i>cpe</i>	HindIII	PCR1 1213 bp	PCR2 1697 bp	PCR3 1397 bp
NCTC 8239	A	+	+	multiple	+	+	+
1090	A	+	+	3	+	+(S1)	+
1995	A	+	+	—	—	—	+
2154†	A	+	—	ND	+(S2)	—	—
2672	A	+	+	multiple	+	+	+
4457	A	+	+	—	—	—	+
4743	A	+	+	1	+	+	+
5387	C	+	—	3	+(S2)	—	—
FD	D	+	—	—	—	—	—

* +, positive result; —, negative result; +(S1), positive result, about 800 bp smaller than expected; +(S2), positive result, about 400 bp smaller than expected; ND, not determined.

† Has been enterotoxin positive (Skjelkvåle *et al.*, 1979).

Table 4. Primers and probes used in the study of the architecture of the DNA 5' of the *cpe* on different strains of *C. perfringens*

Primer names*	Position of sequences	Primer sequences
PCR1 F	377–397	AAGCTATTGGAGAGACATCT
PCR1 R	1590–1569	TGAAAACCGTGAAGAATTTGG
PCR2 F	1221–1241	TCCTTTCGGGATAAAACGC
PCR2 R	2918–2898	TGCTACACTTAAAAGTGTCC
PCR3 F	2773–2793	ACATTCTGGTGTATGGGAT
PCR3 R	4170–4151	CATTTTCGAACACATTGGA

* R, reverse; F, forward.

like transition state regulator seems to be present in *C. perfringens* and fully conserved Hpr consensus binding sequences appear both 5' and 3' to *cpe*. It might be argued that such sequences may appear at random in DNA with high AT content. However, we have searched among the 34 different *C. perfringens* genes present in the EMBL database, and have not come across a single gene with an Hpr-binding sequence in pairs, or both up- and downstream of a gene. The DNA region containing the 5' sequence is fully conserved in terms of PCR fragment size between all enterotoxin-positive strains tested. Thus, a mechanism of Hpr-linked repression of *cpe* expression until entry into the transition state would seem to be indicated. Even in *B. subtilis*, however, where lowering of *hpr* expression is observed upon entry into the transition state, the mechanism of control is only partly understood (Strauch & Hoch, 1993). It will be especially interesting to develop these studies in parallel for sporulation within the anaerobic clostridia.

REFERENCES

- Alouf, J. E., Knöll, H. & Köhler, W. (1991). The family of mitogenic, shock-inducing and superantigenic toxins from staphylococci and streptococci. In *Sourcebook of Bacterial Protein Toxins*, pp. 367–414. Edited by J. E. Alouf & J. H. Freer. London: Academic Press.
- Altschul, S., Gish, W., Miller, W., Myers, E. & Lipman, D. (1990). A basic logical alignment search tool. *J Mol Biol* **215**, 403–410.
- Ansorge, W., Sproat, B. S., Stegeman, J. & Schwager, D. (1986). A non radioactive automated method for DNA sequence determination. *J Biochem Biophys Methods* **13**, 315–323.
- Ansorge, W., Sproat, B. S., Stegeman, J., Schwager, D. & Zenke, M. (1987). Automated DNA sequencing: ultrasensitive detection of fluorescent bands during electrophoresis. *Nucleic Acids Res* **15**, 4593–4602.
- Bowness, P., Moss, P. A., Tranter, H., Bell, J. I. & McMichael, A. J. (1992). *Clostridium perfringens* enterotoxin is a superantigen reactive with human T cell receptor Vb6.9 and Vb22. *J Exp Med* **176**, 893–896.
- Canard, B., Saint-Joanis, B. & Cole, S. T. (1992). Genomic diversity and organization of virulence genes in the pathogenic anaerobe *Clostridium perfringens*. *Mol Microbiol* **6**, 142–1429.
- Duncan, C. L., Strong, D. H. & Sebald, M. (1972). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *J Bacteriol* **110**, 378–391.
- Gibert, I., Carroll, K., Hillyard, D. R., Barbé, J. & Casadesus, J. (1991). IS200 is not a member of the IS600 family of insertion sequences. *Nucleic Acids Res* **19**, 1343.
- Granum, P. E. (1990). *Clostridium perfringens* toxins involved in food poisoning. *Int J Food Microbiol* **10**, 101–112.
- Granum, P. E. & Stewart, G. S. A. B. (1993). Molecular biology of *Clostridium perfringens* enterotoxin. In *Genetics and Molecular Biology of Anaerobic Bacteria*, pp. 235–247. Edited by M. Sebald. New York: Springer.
- Granum, P. E., Telle, W., Olsvik, O. & Stavn, A. (1984). Enterotoxin formation by *Clostridium perfringens* during sporulation and vegetative growth. *Int J Food Microbiol* **1**, 43–49.

- Hanahan, D. (1983).** Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Hanna, P. C., Wnek, A. P. & McClane, B. A. (1989).** Molecular cloning of the 3' half of the *Clostridium perfringens* enterotoxin gene and demonstration that this region encodes receptor-binding activity. *J Bacteriol* **171**, 6815–6820.
- Iwanejko, L. A., Routledge, M. N. & Stewart, G. S. A. B. (1989).** Cloning in *Escherichia coli* of the enterotoxin gene from *Clostridium perfringens* type A. *J Gen Microbiol* **135**, 903–909.
- Kallio, P. T., Fagelson, J. E., Hoch, J. A. & Strauch, M. A. (1991).** The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein. *J Biol Chem* **266**, 13411–13417.
- Kristensen, T., Voss, H., Schwager, J., Stegemann, J., Sproat, B. & Ansorge, W. (1988).** T7 DNA polymerase in automated dideoxy sequencing. *Nucleic Acids Res* **16**, 3487–3496.
- Labbe, R. G. & Duncan, C. L. (1977).** Evidence for stable messenger ribonucleic acid during sporulation and enterotoxin synthesis by *Clostridium perfringens* type A. *J Bacteriol* **129**, 843–849.
- Pearson, W. & Lipman, D. (1988).** Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**, 2444–2448.
- Perego, M. & Hoch, J. A. (1988).** Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. *J Bacteriol* **170**, 2560–2567.
- Pitcher, D. D., Saunders, N. A. & Owen, R. J. (1989).** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Rosenberg, A. H., Simon, M. N., Studier, W. F. & Roberts, R. J. (1979).** Survey and mapping of endonuclease cleavage sites in bacteriophage T7 DNA. *J Mol Biol* **135**, 907–915.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sebald, M. (1993).** *Genetics and Molecular Biology of Anaerobic Bacteria*. New York: Springer.
- Sherratt, D. (1989).** Tn3 and related transposable elements: site specific recombination and transposition. In *Mobile DNA*, pp. 163–184. Edited by D. E. Berg & M. M. Howe. Washington, DC: American Society for Microbiology.
- Skjelkvåle, R. & Duncan, C. L. (1975).** Enterotoxin formation by different toxigenic types of *Clostridium perfringens*. *Infect Immun* **11**, 563–575.
- Skjelkvåle, R., Stringer, M. F. & Smart, J. L. (1979).** Enterotoxin production by lecithinase-positive and lecithinase-negative *Clostridium perfringens* isolated from food poisoning outbreaks and other sources. *J Appl Microbiol* **47**, 329–339.
- Strauch, M. A. & Hoch, J. A. (1993).** Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Mol Microbiol* **7**, 337–342.
- Stewart, G. S. A. B., Lubinsky-Mink, S., Jackson, C. G., Cassel, A. & Kuhn, J. (1986).** pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. *Plasmid* **15**, 172–181.
- Van Damme-Jongsten, M., Wernars, K. & Notermans, S. (1989).** Cloning and sequencing of the *Clostridium perfringens* enterotoxin gene. *Antonie Leeuwenhoek* **56**, 181–190.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Received 23 June 1993; revised 13 September 1993; accepted 15 September 1993.