Characterisation of an enterotoxin-negative, cytotoxin-positive strain of *Clostridium sordellii*

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In ileal loop assay, ELISA and anion-exchange column chromatography, *Clostridium sordellii* strain 6018 was shown to produce a cytotoxin, but no detectable enterotoxin. DNA sequence and polymerase chain reaction analyses indicated that the lack of enterotoxin activity is not due to a lack of gene transcription, but to lack of a major portion of the enterotoxin gene. This is the first characterisation of such a strain.

Introduction

Clostridium sordellii causes gas gangrene in man, cattle and sheep [1–3]. This gram-positive anaerobic bacterium produces a haemorrhagic enterotoxin (H) and a lethal cytotoxin (L), which have been purified, characterised and shown to be immunologically similar to *C. difficile* enterotoxin A and cytotoxin B, respectively [4–7]. On the genome of *C. difficile* the cytotoxin gene is separated from the enterotoxin gene by a short open reading frame (ORF) which is flanked by short intergenic regions. It was originally thought that non-toxigenic strains of *C. difficile* lack the genes for both enterotoxin and cytotoxin [8–11], but in more recent studies a *C. difficile* strain that produces a modified cytotoxin and lacks the entire 3' portion of the enterotoxin gene and part of the 5' portion has been found [12–14]. Furthermore, *C. difficile* strains of serogroup F possess cytotoxin and enterotoxin genes, but do not produce an enterotoxin and produce a modified cytotoxin [15].

C. sordellii has received less attention. Martinez and Wilkins [7] and Popoff [6] showed that *C. sordellii* strains VPI 9084 and IP 82 produce a cytotoxin and an enterotoxin, and we have studied *C. sordellii* 6018 cytotoxin [4, 5, 16] and its gene [17]. This study examined the failure of *C. sordellii* strain 6018 to produce an enterotoxin and the absence of a major part, or possibly all, of the enterotoxin gene.

Materials and methods

Bacterial strains and growth

C. difficile 68750 and *C. sordellii* 6018 are clinical isolates from this clinical laboratory, and *C. sordellii* VPI 9048 was included as a toxin-positive control. Cultures were grown anaerobically at 37°C in Brain-Heart Infusion Broth (Difco) for 72 h for protein studies and for 24 h for DNA analyses.

Enterotoxin and cytotoxin assays

Cell-free supernates from clostridial cultures were obtained by centrifugation (5000 g, 5 min) and filtration through a 0.45 µm membrane filter. One ml was injected into the ileal loop of an anaesthetised rabbit. Fluid accumulation was measured 18 h later. Positive (*Escherichia coli* LT EWD 299, kindly supplied by Dr S. Falkow) and negative (phosphate-buffered saline) controls were included [18]. Enterotoxin was also assayed with the ToxA ELISA test (Biowhittaker, France) [19]. Cytotoxin assays were performed on McCoy cells cultured at 37°C in a humidified atmosphere of CO₂ 5%, air 95% [19].

Chromatography

Proteins from a further 500 ml of the cell-free supernate were separated by anion-exchange chromatography on a MonoQ HR column, with 0–1 M NaCl gradient elution [4]. Fractions were assayed for cytotoxin activity. Cytotoxin-positive fractions were re-chromatographed and re-tested for cytotoxicity.

DNA extraction and PCR analysis

DNA was isolated from 5 ml of culture [20] and 50 ng were amplified in a total volume of 100 µl [10]. Primers developed for the *C. difficile* enterotoxin gene [10]

AAAAAGGTAATAATATATGCGTTTATAAATAAAAGAATAAAAATAAAGGAATAAAGATTTTATTCTACAGTCCTATTCTCAAAAATAATTAATATAAACCTAATTTCAA 120

CAAATATCAACAGTAACCTATGC AATTAAAGCTATATAAGTTGGATTGTATAATAACAAGCTGAAAGTGAAATAATAGATAAAAAAATAAAGCTGCCAAATAGTTTTAAATAATAAT 240

TCAAAAAAATAAAAGTATGTTTACATATTCATATAAAAAATAATCTTACTTTATGTAAAGTCAGTTAACTTAACCTTATTTGAAATAAAAAATGTTAGGAGGGAATAATATGAAACCT 360

TAAATCTCAAAAAATTTAATATCAATATACCTTTTCATAAATAAGAGAAGAATAATAAGGTATTTTACAATTTAGATATAAGTGCTAAAAAAGATGTTATATAAAGGGGGAAATTTAATGAA 480

CTTAGTTAACAAAGCCCAATTACAAAAAATGGTATATGTA AAAATTTTCGATTCAAGAAGATGAGTACGTAGCAATATTAATGCTCTAGAAGAATATCACAACATGTCAGAAAGTAGTG 600

L V N K A Q L O K M H Y V K F R I O E D E Y V A I L N A L E S Y E H N M S E S S V
AGTTTGAAAGTATTTAAATTAAGCATATAAATAATCTCACAGATAATTTACCCTGAACADATATAAAATCTGGAAGGAATAAAGCCTTAAAAAATTTAAAGAAATATCTCAATATGGA 720

V E K Y L K L K D J N N L T D N Y L N T Y K K S G R N K A L K K F K E Y L T H E
AGTTATAGAGCTAAAAAATAATAGTCTAACTCGCAAGAAAAATTTTATATGGATTGGAGGACAAATAAGTATACCGCTATCAACTATATAATCAATGGAAAGATGAA 840

V L E L K N N S T L P V E K E N L H F I W J G G O I N D T A I N Y J N O W K D V N
TAGCGATTATACAGCTAAAGTTTTTATGATAGTAATGCATTTTGTATAATACATTAAGAAAACTATTGTTGAGTCAGCAACAAATAATACTCTTGAGTCATTAGACAAAACTTAA 960

S D Y T V K V F Y D S N A F L I N T L K K T I V E S A T N N T L E S F R E N L H
TGACCCTGAATTCGATTATAATAATTTTATAGAAAACCTTGGAATAATATATGATAAACA AAAAATCTTTATAGATTATTAAGCTCTCAGATAGAGAAGATCTCGAATTTTAAAT 1080

D P E F T D Y N K F C Y F Y R K R M E I J Y D K O K H F I D Y Y K S Q I E E N P E F I I
TGATAATTAATAAAAACTATCTCTCAAAATGAGTATTCAAAGACCTAGAAGCCCTTAATAAGTATGTGAAGATCTTTAAATAAAATTAAGCTCTAATAATGGTAATGATATGATACAGAA 1200

O N I I K T Y L S N E Y S K D L E A L N K Y I E E S L N K I T A N N G N D I R N
TCTAGAAAAATTTGCTGATGAGGATTTGCTAAGATTATATAATCAAGAATTAGTAGAAGATGGAATTTGGCTGCTGCTATGATATAGCAATATCTATGTTAAAGAAGATGGTGG 1320

L E K F A D E D L V R L Y M Q E L V E R W N L A A A S D I L R I S H L K E D G G
TGATATTTTAGATGTTGACATCTTACAGGATATACACAGCATTTTAAATCTATAAACAAGCTGATTTCGATAACAAATACAAGTTGGGAAGATGATAAAGTTACAGCGCTATAATCAA 1440

V Y L D V D I L P G I O P D L F K S I N K P D S I T N T S W E H I K L E A I H K
ATATAAGGAATATACAGGATACAGTCAAGAATAATTTGACATGTTAGTGAAGAAGTTCAGCGCAATTTGAATCTGCTTTAAGTTCTCAATCAGATAGTCAAGAAATTTTTTGCC 1560

Y K E Y I P C Y T S K N F D M L D E E V O R S F E S A L S S K S D K S E I F L P
ACTTGATGATATAAAGTATCCCGCTTAGAAGTAAAAATGCAATTTGCCAAEACTCTGTTATAAATCAAGCCTTAATTTCTTTAAAGATTCCTATTGTAGCTATTAGTAATAATCA 1680

L D D A I K V S P L E V P K I A F A N N S Y I N O A L I S L K D S Y C S D L V I N O
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I K N R Y K J L N D N L N P S I N E G T D F N T T H K I F S D K L A S I S N E D
TAATATGATGTTTATGATAAAAAATTAACAATTTTAAAGTTGGATTTCGCTCAGATGTTAGAAAGTACTATTAACCTAAGTGCACTGGAGTATATACAGGAGCTTATCAAGATTTTGT 1920

N H M F H A I K T T C N Y L K V G F A P D Y R S T I N L S G P G V Y T G A Y O D L L
AATGTTTAAAGATATAGTACAAATTTCTNTTACTAGAACCTGAGTTAAGAATTTTGAGTTTCTCTAAAATTTCTCTAAGTACAGAAAGCAAGAAATACTAGTTTATTTGGTCATT 2040

M F K D N S T N I H L E P L R M F E F P K T I S Q L T E Q E A C I G T S L V S F
TAACCAGCAAGAGCTCAAGTCTCAATTTGCAAGAATATAAAAAAGGTTATTTTGAGGTGCATCTGGAGAAGATGATAATCTTGATTTTGGCTCAAAATACAGTACTTGATAAAGATTATGT 2160

H O A R A K S O F E E Y K K G Y F E G A L G D N L D F A O N T L V L D K D Y V
TTCTAAAAAATATTATCATCAATGAAAACCGAAATAAGAAATATATTCATTATTTGTCACCTACAAGGAGATAAAACTCAGCTATGAAGCATCATGTAACCTTTTCTTCAAAAGATCC 2280

S K K I L S S M K T R N K E Y I H Y I V O L G D K I S Y E A S C N L F S K D P
TTATCTAGTACTATATCAGAAAATATAGAAGGTCAGAAACAGCATTTACTATTATTTGTCAGATGCTCAGATATAAGAAATAGATAAATATAGAATTTCCATATCAAAATTTCTCAA 2400

Y S S I L Y O K N I E G S E T A Y Y Y V A D A E I K E I D K Y R I P Y O I S N
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K R N I K T L F I G H G K S E F N T D T F A N L D V O S L S S E I E T I L N L A
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K A D I S P K Y I E I N L L G C N H F S Y S I S A E Y Y P G K L L L K I K D R
AGTATCAGAATTAATGCCATCTAAGTCAAGACTCTTATACAGTAAGTGCAATCAATATGAAGTTAGAATAAATGAAGAGGAAAAAGAAATTTAGATCATTTCTGTAATGCAAT 2760

V S E L M P S I S O D S I T Y S A N O Y E V R I N E E G K R E I L D H S G K W I
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N K E E S I J K D I S S K E Y I S R P K E N K I Y K S K Y L H E L S T L L O
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E J R N N A N S S D I D L E K K V M L T E C E I N V A S N I D R O I V E G R I E
AGAAGCTAAAAATTTGACTCTGACTCTTAATATATAAAAAATGAATTTAAAGTAATAGCAATTTCTGATTCAATTATGATTAAACACTCAAAATGGATAGATAGATTTCTCA 3120

E A K N L T S D S I N Y I K N E F K L I E S I S D S L Y D L K H O N G L D C D S H
TTTTATCTCTTTTGAGSATATCTCCAAGACTGAAAATGGATTTAGGATAAGGTTCAATTAAGAAACTGGAAGCTCTATATTTAGAAACTGAAAAGAAATTTCTCTGAATATGC 3240

F I S F E D I S K T E N G F R I R F I N K E T G N S I F I E T E K E I F S E Y A
TACTCATATATCTAAGAAATTTCTAATATAAAGATACTATTTTGATAATGTAAATGGCAAAATTAGTAAAAAAGTAAATCTAGATGCTGCACATGAAGTAAATACTCTAAATTTCTGC 3360

T H I S K E I S N I K D T I F D H N V N K L V K K V N L D A H E V N T L N S A
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F F I Q S L I E Y N T T K E S L S N L S V A H K V Q V Y A O L F S T G L N T I T
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D A S K V V E L Y S T A L D E T I D L L P T L S E C L P I I A T I I D G V S L G
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A A I K E L S E T N D P L L R O E I A G A I K I G I H A V N L T A A S T A I V T S A
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Y S L S L S P Y N M N I D N L V E N D T W V I D V D N V V K N I T I E S D E I
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O K G E L I E N I L S K L N I E D N K I L N N H T I N F Y G D I N E S N R F

A N Y I N E K I N V N I N D L S I R Y V W D N D G S D L I L I A N S E E D N O P
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 A A Y S D G C F F D Y E F G L V S L D N D Y F Y I N S F G N H V S C L I Y I N D S
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 L Y Y F K P P K N N L I T G F T T I D G N K Y Y F D P T K S G A S I G E I T I
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 D G K D Y Y F N K O G I L Q V G V I N T S D G L K Y F A P A G T L D E N L E G E
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 S V N F I G K L N I D G K I Y Y F E D N Y R A A V E W K L L D D E T Y Y F N P K
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 T G E A L K G L H O I G D N K Y Y F D D N G I M O T G F I T I N D K Y F Y F N N
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 D G V M O V G Y I E V N G K Y F Y F G K N G E R O L G V F N T P D G F K F F G P
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 S E
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 I R K I A M I A C I F F L V D M L T O F N F L F I L P Q E Y I N F F R F O H
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 L L Y T I T D E V K I E D R
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 TATATTACAGAAATCATTTTGTCAACATATATTTTAAACCATCTATACCAAGAAATATATAGCCCCATCGATAGTAAATATCCCTCATCTGCACTATAGCAGTATCAGGCATAAA 9000
 GTAATAGCTAATACATTAATGGTATCGTTTATTCGATTGATATTAGATTACTAATGAATAGAATCGTATATAGTATTTAAACCAGTACTAAATAGTTGAGCATAAAGTTGGACCTTAA 9120
 CCTAAGTGCTTAAATTTTAAATACATATTTGTTGCTACTATAATCTATTTCATGATTGAATAAAGATGCTGAATTTAATGTATTAATGAGGTACATATTCTAATCTATATTCCTT 9240
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 TCTTTAATTTGTTGATCCAATGAACAAAGTTTTTGTATGATAATTATATATATTTCTTATCTTCAATAGCCTATAATTTTATTTTCCCAACTATCTACTCCGATAGAGCTA 9600
 GGCTAGA 9608

Fig. 1. Nucleotide sequence of a 9.6-kb fragment of *C. sordellii* genomic DNA containing the cytotoxin gene and a short ORF: putative ribosomal binding sites are indicated (●), putative '−10' and '−35' sequences are underlined.

(5'-GAAGCAGCTACTGGAT GGC-3' and 5'-AGCA-GTGTAGTATTAATA G-3'), were used to amplify the *C. sordellii* enterotoxin gene.

Cloning and sequence analysis

All cloning techniques employed were as described by Sambrook *et al.* [21]. An oligonucleotide probe (5' GATGATATAAAATATTATTTTGGAT 3', termed 'oligo rep') corresponding to a repetitive region at the 3' end of *C. difficile* cytotoxin was used as a probe to clone a 3.4-kb *HindIII*/*EcoRI* fragment of *C. sordellii* genomic DNA. Oligonucleotides corresponding to the

5' and 3' ends of this fragment were used to clone overlapping fragments. Two successive oligonucleotide probes were necessary at the 5' end in order to cover the entire *C. sordellii* cytotoxin gene and its flanking regions, 9.6 kb in all. Fragments were cloned in pUC18 or pCRII and were subcloned into M13 mp130 or mp131 for sequencing. The *E. coli* host used was TG1 for pUC and M13 cloning, whereas *E. coli* strain inv α F' was used with pCRII. Sequencing was performed with the Pharmacia T7 sequencing kit. All restriction sites used for subcloning were sequenced across. Sequence data were analysed with DNASTar Software (DNASTar Ltd, London) for the Macintosh Computer.

Table 1. Characteristics of *C. difficile* 68750, *C. sordellii* VPI 9048 and *C. sordellii* 6018

Bacterial strain	Culture supernate cytotoxicity	Enterotoxigenicity (ileal loop assay)	ELISA for <i>C. difficile</i> enterotoxin	PCR with <i>C. difficile</i> enterotoxin-specific primers
<i>C. difficile</i> 68750	+	+	+	+
<i>C. sordellii</i> VPI 9048	+	+	+	+
<i>C. sordellii</i> 6018	+	—	—	—

Results

The nucleotide sequence obtained for a 9.6-kb fragment of *C. sordellii* genomic DNA is presented in Fig. 1. The first long ORF corresponds to the cytotoxin and the second short ORF is unidentified. *C. sordellii* cytotoxin shows high homology with *C. difficile* cytotoxin (76% identity, 90% similarity), and the short ORF shows 73% identity and 93% similarity with its *C. difficile* counterpart.

No enterotoxin activity was found in *C. sordellii* 6018 culture supernates in the rabbit ileal-loop assay (Table 1), whereas enterotoxin activity was detected for *C. difficile* 68750 and *C. sordellii* VPI 9048.

C. difficile and *C. sordellii* enterotoxins are also cytotoxic, but to a lesser degree than the cytotoxins [7]. Culture supernates for *C. difficile* 68750 were cytotoxin-positive and two peaks of cytotoxicity were obtained in anion-exchange chromatography eluting between 0.13 and 0.32 M NaCl (enterotoxin) and at 0.5–0.6 M NaCl (cytotoxin). *C. sordellii* VPI 9048 gave similar results (0.22 M NaCl, enterotoxin and 0.38–0.51 M NaCl, cytotoxin). *C. sordellii* 6018 yielded a single peak eluting at 0.38–0.43 M NaCl, corresponding to the cytotoxin.

Culture supernates of *C. difficile* 68750 and *C. sordellii* VPI 9048 gave positive results in the enterotoxin assay, as did the fractions corresponding to the first cytotoxic peak eluted in anion-exchange chromatography. In contrast, *C. sordellii* 6018 culture supernate and anion-exchange fractions eluting between 0.1 and 0.3 M NaCl showed no detectable enterotoxin in ELISA.

PCR amplification for *C. difficile* 68750 resulted in multiple bands characteristic of the repetitive region of the enterotoxin gene [10]. Similarly, PCR amplification of *C. sordellii* VPI 9048 DNA resulted in a multiple band pattern, distinguishable from that for *C. difficile*. In contrast, no amplification was found with these primers for DNA isolated from *C. sordellii* 6018 (Fig. 2).

Discussion

In *C. difficile* the cytotoxin gene is separated from a short ORF by 122 bp, which in turn is separated from

the enterotoxin gene by 726 bp [22]. Sequencing of the cytotoxin gene from *C. sordellii* 6018 showed a short ORF 210 nucleotides downstream. However, sequences for the next 1400 nucleotides downstream did not reveal another ORF that could correspond to the *C. sordellii* enterotoxin gene (EMBL accession number, X82638). Furthermore, no sequence homology was found with the recently published NH₂-terminal peptide sequence of the enterotoxin [7]. Therefore, *C. sordellii* 6018 might: (1) produce an active enterotoxin but the small ORF-enterotoxin gene intergenic region may be longer than for *C. difficile*; (2) possess an enterotoxin gene that is transcribed but the enterotoxin is inactive; (3) possess an enterotoxin gene that is not transcribed or (4) not possess an enterotoxin gene, i.e., the gene could be partially or completely absent. Rabbit ileal loop assay indicated that this strain does not produce an active enterotoxin. The ELISA assay for *C. difficile* toxin showed strong cross-reaction for the enterotoxin of *C. sordellii* VPI 9048, but no reaction with supernates or fractions from *C. sordellii* 6018. Neither of these results eliminates the possibility that either an active enterotoxin is produced, but at levels lower than can be detected, or that an inactive, non-cross-reacting protein is produced. However, together with the DNA sequencing evidence, and the failure to detect amplification with the *C. difficile* enterotoxin primers, the results strongly indicate that this strain contains a large deletion covering most, if not all, of the enterotoxin gene.

Fig. 2. PCR amplification of *Clostridium* DNA: 1, *C. difficile* 68750; 2, *C. sordellii* 6018; 3, *C. sordellii* VPI 9048.

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