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Clostridium spiroforme Toxin Genes are Related to *C. perfringens* Iota Toxin Genes but have a Different Genomic Localization

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Summary

Clostridium spiroforme toxin belongs to the iota toxin family and consist of an ADP-ribosyltransferase component (Sa) and a binding component (Sb) which is involved in the binding and internalization of the toxin within the cell. In this study, the respective genes, *sas* and *sbs*, were characterized. The genes are orientated in the same way as the *C. perfringens* iota toxin genes and the *C. difficile* ADP-ribosyltransferase (CDT) genes. The *sas* gene is located upstream of the *sbs* gene. The genes are transcribed in the same orientation, and are separated by a short (41 bp) non coding sequence. The encoded proteins Sa and Sb are similar (about 80% identity) to the iota toxin and CDT, and these toxins have the same domain organization. In contrast to the iota toxin genes which are present on large plasmids in *C. perfringens* E, *C. spiroforme* toxin and CDT genes are present on the chromosome in the *C. spiroforme* and *C. difficile* strains analyzed, although these strains also contain large plasmids. A variation of the iota toxin sequences (80% identity) was observed in 3 of 4 *C. perfringens* E strains, and one strain had only the enzymatic component gene.

Key words: iota toxin – ADP-ribosylation – *Clostridium spiroforme* – *Clostridium perfringens* – *Clostridium difficile*

Introduction

The actin cytoskeleton is a target for several bacterial toxins. Large toxins such as *Clostridium difficile* toxin A and toxin B, *C. oedematiens* toxin A, *C. sordellii* lethal toxin and the C3 enzymes of *C. botulinum* and *C. limosum* modify regulatory proteins from the Rho family which are involved in the control of the actin cytoskeleton assembly, by monoglucosylation or ADP-ribosylation (JUST et al., 1995; JUST et al., 1995; SELZER et al., 1995; JUST et al., 1996; POPOFF et al., 1996). The clostridial binary toxins (*C. perfringens* iota toxin, *C. spiroforme* toxin, an ADP-ribosyltransferase from *C. difficile* (CDT), and *C. botulinum* C2 toxin) modify monomeric actin by ADP-ribosylation causing disruption of actin filaments (AKTORIES et al., 1986; SIMPSON et al., 1987; VANDEKERCKHOVE et al., 1987; POPOFF et al., 1988). The binary toxins consist of two separate protein chains. The binding component (ca. 100 kDa) recognizes a cell surface receptor and allows the internalization of the enzymatic component (ca. 45 kDa) into the cytosol (CONSIDINE et al., 1991). *C. perfringens* iota toxin, *C. spiroforme* toxin and CDT (iota toxin family) are immunologically related and cross react weakly with C2 toxin (POPOFF and BOQUET 1988; POPOFF et al., 1989). Functional complementation also occurs between the iota toxin family components. The binding component of iota toxin and *C. spiroforme* toxin (Ib and Sb respectively) can be interchanged with the corresponding enzymatic components (Ia and Sa) and with that of CDT (CDTa) to form fully active toxins (POPOFF and BOQUET 1988; SIMPSON 1989). The iota toxin genes *(iap* and *ibp)* and the CDT genes *(cdtA* and *cdtB)* have been characterized (PERELLE et al., 1993; PERELLE et al., 1997).

To elucidate the structure of the *C. spiroforme* toxin, we characterized its genes, and compared their organization with iota toxin and CDT genes. To clarify the mode of transfer of the *Clostridium* iota toxin family genes we determined their localization and analyzed their environment and the presence of insertion sequences (IS*1151*

and IS*200*-like element) which may be involved in mobilization of toxin genes (DAUBE et al., 1993; BRYNESTAD et al., 1994).

Materials and Methods

Bacterial strains and DNA: *C. perfringens* E strains NCIB10748, NCTC8084, 46088 and CN5065, *C. perfringens* D strain 945P, *C. spiroforme* NCTC11493, CS246, and *C. difficile* CD196 were grown in broth containing Trypticase (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine-HCl (0.5 g/liter) (pH 7.2) under anaerobic conditions.

pUC19 (Appligene, Strasbourg, France) was used for cloning in *Escherichia coli* TG1.

Clostridium DNA preparation: Total DNA was extracted from *Clostridium* harvested of an 6 h culture as previously described (POPOFF et al., 1985).

Plasmid DNA from *Clostridium* was prepared by a modification of the method of KIM and BLASCHEK (KIM et al., 1989). Briefly, bacteria from 500 ml overnight culture were suspended in 10 ml of 10 mM Tris-HCl (pH 8)-10 mM EDTA-20% sucrose containing 10 mg lysozyme and incubated for 30 min at 37 °C. Bacteria were lysed by the addition of 20 ml of 0.2 M NaOH-1% SDS. The mixture was incubated for 5 min in ice and neutralized by the addition of 40 ml 1.5 M sodium acetate (pH 4.6). The suspension was incubated for 1 h on ice and centrifuged (10,000 × g for 20 min). The supernatant was precipitated using 1 volume of isopropanol 2 and centrifuged. Then, the pellet was solubilized in 8 ml 10 mM Tris-HCl (pH 8)-1 mM EDTA, and the DNA was isolated by a cesium chloride gradient centrifugation.

PCR amplification: One hundred nanograms of DNA was amplified by the polymerase chain reaction (PCR) in a total volume of 100 µl as previously described (PERELLE et al., 1993). Primers used to amplify gene specific probes are indicated in Table 1.

Probes and hybridization conditions: Uncut and digested DNAs were separated on a 0.8% agarose gel in Tris, acetate, EDTA buffer (SAMBROOK et al., 1989) run at 50 V overnight. The DNA was transferred onto a nylon membrane (Hybond N+; Amersham, Paris, France). PCR amplified fragments were labeled as hybridization probes using the Megaprime kit (Amersham) and ^{32}P-dATP. The hybridizations were carried out with the Rapid Hybridization Buffer (Amersham) overnight at 50 °C. The membranes were washed in 0.1% SSC-0.1% SDS (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50 °C for 2 h.

Other molecular biology techniques: Ligation and preparation of plasmid DNA from *E. coli* were conducted as described in (SAMBROOK et al., 1989). DNA was sequenced by the dideoxy chain termination procedure using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

Nucleotide sequence accession number: The nucleotide sequence reported in this study has been submitted to the EMBL Data Library with accession number X97969.

Results

Cloning of *C. spiroforme* toxin genes

The recombinant plasmids pMRP44 and pMRP105 (PERELLE et al., 1993) carrying the *iap* and *ibp* genes o. *C. perfringens* were used as probes in the cloning of three DNA fragments (pMRP167, pMRP149, and pMRP229) from *C. spiroforme* CS246 (Fig. 1). pMRP256 and pMRP264 resulted from PCR amplified fragments using the primers P356–P395, and P222–P396 respectively, and cloned into pUC18. The sequences of the primers were deduced from the DNA sequencing of pMRP149 and pMRP229, except P222 which is a degenerate primer deduced from an internal peptide sequence of Sa (PERELLE et al., 1993). The 5' sequence of *sas* was obtained by inverse PCR (HUANG et al., 1993) using *C. spiroforme* DNA digested by *Nsi*I and the primers P413 and P438 (Fig,. 1). The amplified DNA fragment ligated into pUC18 was unstable in *E. coli*, and therefore it was sequenced directly. The sequenced region of 4465 bp had two open reading frames named *sas* and *sbs* (Fig. 2).

Characterization of *C. spiroforme* toxin genes and proteins

• ***sas* gene and Sa protein:** The *sas* gene was precedec by a ribosome binding site (GGAG) and encoded a protein of 459 amino acids (52,523 Da) (Fig. 2). A consen-

Table 1. PCR primers used for amplification of gene probes. a. P333 corresponds to the inverted repeat of IS*1151*.

Gene	PCR Primers	Size of amplification	Gene sequence reference
iap	P245 ATGGCTTTTATTGAAAGACCAGAA P246 TCATATTTTACACTTCCGAAT	425	(37)
ibp	P256 ATGGAAGAAATAACAAATGAAAATACAC P257 TTAATTAACACTAAGCACTAATAAC	2507	(20)
cpe	P145 GAAAAGATCTGTATCTACAACTGCT P146 GCTGGCTAAGATTCTATATTTTTG	246	(20)
IS*1151*	P333 CATGGCCGTCAACCTAAGAAG[a]	1689	(9)
IS*200*-like	P334 ATGCTTGTTGAAATACCACCTAAA P335 CTTGCTACACTTAAAATGTCCATA	268	(4)

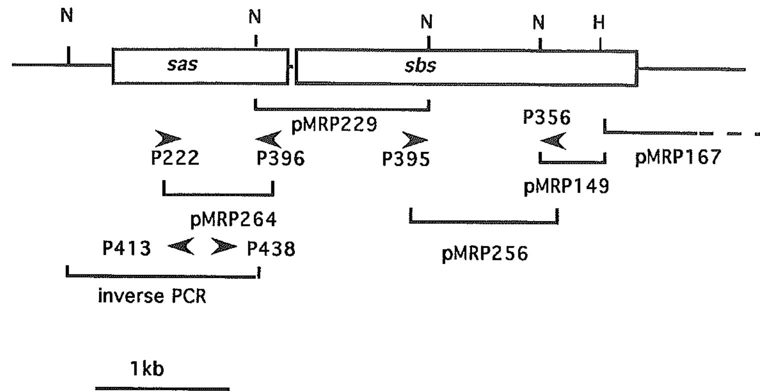

Fig. 1. Cloning strategy for *C. spiroforme* toxin genes *sas* and *sbs*. Arrows correspond to primers used for PCR amplification. H, *Hin*dIII; N, *Nsi*I.

sus promoter sequence could not be identified in the upstream sequence. The coding sequences of Sa, Ia and CDTa were found to be very similar (81.7% to 84.6% identity), whereas the 5' region of *sas* is weakly related (44.2% to 60%) to the corresponding sequence of *iap* and *cdta* genes (PERELLE et al., 1993; PERELLE et al., 1997). The *iap* promoter region has an inverted repeat at the transcription start site and three binding motifs to an Hpr-like regulator (manuscript in preparation). These features were not found in the upstream region of *sas*.

The predicted 44 N-terminal amino acids of the Sa protein forming a hydrophobic segment flanked by charged residues (Lys-2, Lys-3, Lys-5, Asp-36 and Arg-38) could correspond to a signal peptide. A common cleavage site for bacterial signal peptidases is Ala-x (VON HEIJNE et al., 1989). Therefore, it was predicted that Ala-44-Asn-45 may be a putative cleavage site. The predicted physicochemical properties (47,429 Da, pI 6.15) of the secreted Sa which comprises 415 amino acids, are in agreement with those determined experimentally (44 kDa, pI 6.2) (POPOFF et al., 1989).

- ***sbs* gene and Sb protein:** The *sbs* gene was located 41 nucleotides downstream from the *sas* gene and was preceded by a consensus ribosome binding site sequence (GGAGG) localized 7 bp upstream from the start codon (Fig. 2). The encoded protein by *sbs* consisted of 879 amino acids and had 3 domains. The 44 N-terminal amino acids form an hydrophobic segment with 3 charged residues at the terminal end. This was probably a signal peptide. A putative cleavage site for signal peptidase was localized between Lys-44 and Thr-45. The N-terminal sequence of the naturally activated form of Sb determined by protein sequencing (GWGDEDLD) (PERELLE et al., 1993) was identical to amino acids Gly-216 to Asp-223 (Fig. 2). The deduced proteins from sequence between Thr-45 to Ser-215 (171 residues, 19,734 Da) and from Gly-216 to Gln-879 (663 amino acids) were predicted to be the propeptide and the mature Sb respectively, produced from the precursor protein by proteolytic cleavage (POPOFF et al., 1989). The predicted molecular mass (73,986 Da) and pI (4.79) was consistent with results reported for mature Sb (76 kDa and pI 4.7) (POPOFF et al., 1989).

Analysis of the homology between *C. spiroforme* toxin and other related toxins

The secreted Sa and Sb components had similar molecular masses and a high degree of identity (78.8 to 84.2%) with the corresponding components of iota toxin and CDT (PERELLE et al., 1993; PERELLE et al., 1997). The mature components of the iota toxin family are very similar (83 to 85% identity), whereas the signal peptide sequences are divergent (40 to 61% identity). Presumably, the functional domains have been better conserved during evolution. The enzymatic components of the iota toxin family were distantly related to the respective component (C2-I) of C2 toxin (10% identity) (FUJII et al., 1996). This is consistent with the lack of cross immunoreaction between Sa, Ia, CDTa and C2-I (POPOFF and BOQUET 1988), and indicates that C2 toxin was a distinct binary toxin. The binding components Sb and CDTb are related to the binding component of anthrax toxins as it has been reported for Ib (33.9% to 34.6% identity) (PERELLE et al., 1993).

The ADP-ribosylation active site is related to that of other ADP-ribosylating toxins such as Pertussis toxin and Cholera toxin (PERELLE et al., 1996; VAN DAMME et al., 1996). It consists of a NAD binding cavity formed by a β-strand and an α-helix flanked by three residues important for the catalytic activity (Arg-295, Glu-378 and Glu-380) (DOMENIGHINI and RAPPUOLI, 1996; PERELLE et al., 1996). This structure is conserved in Sa, CDTa and C2-I. The Arg-294, Glu-377 and Glu-379 residues of Sa align with the corresponding residues of Ia, CDTa, and C2-I (Fig. 3).

A motif (LKDKE) involved in the actin binding site of several actin-binding proteins (PREKERIS et al., 1996) is conserved in the N-terminal part of mature Sa (positions 9 to 14), Ia (10 to 15), CDTa (14 to 19), and it is slightly different in C2-I (44-LKTKE-48). It may be responsible for the binding of the enzymatic components of the clostridial binary toxins with the actin substrate.

The activated binding components (98 kDa for Sb and Ib, and 94 kDa for CDTb) are produced from the secreted forms by proteolytic removal of a N-terminal propep-

1 ATGCATTCTAATCAAAATCTTTACCTCTTAAACAAAGTCGTATTTACTATATTTTTTTAA

61 AATTTATAATGAAAGTGTAAAGGTTAATAGACGTACCGATATTAACAAAGTTCCCCAGTA

121 AATCCATGGTAATCTTATGGGACTAAGTTAAATGAAAGGAGTGAAATTTATCGTTGATTA

181 AAAAATATTTTGATATTCTCCATAATCTAGGTGATTGTTATAACTGTATTATTGATAAAG

241 TGGAGAACTTTTTATAAATGAATACCCCGCTCTTGTTGGTGGTAGTTTCTTATTTATAAA

301 TAAAATAAGATTTGTTTAAATATATAGACTATTTAGGATTAAATTAAAATTAGGAGTAGA

1 *M K K Y K N N C I S I L L M L F L I L*

360 ACAATGAAAAAATATAAAAATAATTGTATATCTATATTATTAATGCTTTTTCTAATTTTA

20 *T G L F P N T V F A Q G A Q S Y D F R T*

421 ACTGGTTTATTTCCTAATACTGTTTTTGCTCAAGGTGCGCAGAGTTATGATTTTAGAACA

40 *I N N I A* N Y S A I E R P E D F **L K D K**

481 ATCAATAACATTGCCAACTATTCTGCCATAGAAAGGCCAGAAGATTTTCTTAAGGACAAA

60 **E** K A K D W E R K E A E R I E K N L E K

541 GAAAAGGCTAAAGATTGGGAAAGAAAAGAAGCGGAGAGAATAGAAAAAAATCTTGAAAAA

80 S E R E A L E S Y K K D A V E I S K Y S

601 TCCGAAAGAGAAGCCTTAGAGTCGTATAAAAAAGATGCTGTAGAGATAAGTAAATACTCA

100 Q V R N Y F Y D Y P I E A N T R E K E Y

661 CAGGTAAGAAATTACTTTTATGATTATCCGATAGAAGCAAATACTAGAGAAAAAGAGTAT

120 K E L K N A V S K N K I D K P M Y V Y Y

721 AAAGAACTTAAAAATGCAGTATCTAAAAATAAAATAGATAAACCAATGTATGTTTACTAT

140 F E S P E K F A F N K E I R A E S Q N E

781 TTTGAATCCCCAGAAAAATTTGCTTTTAATAAAGAAATAAGAGCAGAAAGCCAGAATGAG

160 I S L E R F N E F K A T I Q D K L F K Q

841 ATTTCCTTAGAAAGATTTAATGAATTCAAAGCAACGATTCAAGATAAACTTTTTAAACAA

180 D G F K D I S L Y E P G N G D K K S T P

901 GATGGATTTAAAGATATTTCTTTATATGAACCAGGTAATGGTGATAAAAAGTCAACTCCG

200 L L I H L K L P K D T G M L P Y S N S N

961 TTACTTATTCATTTAAAATTACCTAAAGATACAGGTATGTTACCATATTCAAATTCTAAT

220 D V S T L I E Q G Y S I K I D K I V R I

1021 GATGTAAGCACATTGATAGAACAGGGATATAGTATAAAGATAGATAAAATCGTTCGTATT

240 V L E G K Q Y I K A E A S V V S C L D F

1081 GTATTAGAAGGAAAACAGTATATAAAAGCAGAAGCTTCAGTTGTGAGCTGTCTTGATTTT

260 K D D V S K G D S W G K A N Y S D W S N

1141 AAAGATGATGTAAGTAAAGGTGATTCTTGGGGAAAAGCTAATTATAGTGATTGGAGTAAT

280 K L S S D E L A G V N D Y M R G R Y T A

1201 AAGTTAAGTTCTGATGAACTTGCTGGTGTAAATGATTATATGCGAGGACGATATACTGCG

300 I N N Y L I A N G P T N N P N A E L D A

1261 ATTAATAACTATTTAATTGCAAATGGTCCTACAAATAATCCCAATGCAGAGCTAGATGCT

Fig. 2/1

Fig. 2. Nucleotide sequence and amino acid translation of the *C. spiroforme* toxin genes. The putative ribosome binding sites are underlined. The predicted signal peptides are shown in italics. Stop codons are indicated by an asterisk. The predicted actin-binding site is in boldface and the predicted ATP/GTP binding site is indicated by dashes.

320 K I N N I E N A L K R E P I P A N L V V
1321 AAAATAAATAATATTGAAAATGCATTAAAACGTGAACCTATTCCCGCTAATTTAGTTGTA

340 Y R R S G P Q E F G L T L S S P E Y D F
1381 TATCGGAGATCTGGACCCCAAGAATTTGGTTTAACTCTTTCTTCTCCTGAATATGATTTC

360 N K V E N I D A F K E K W E G Q T L S Y
1441 AATAAAGTGGAAAATATAGATGCATTCAAGGAAAAATGGGAAGGACAAACGCTATCATAT

380 P N F V S T S I G S V N M S A F A K R K
1501 CCAAATTTTGTCAGCACTAGTATTGGTAGTGTAAATATGAGTGCTTTTGCTAAAAGAAAA

400 I V L R I S I P K N S P G A Y L S A I P
1561 ATAGTACTACGTATAAGTATACCTAAAAATTCCCCTGGTGCTTATTTATCAGCTATTCCA

420 G Y A G E Y E V L L N H G S K F K I S K
1621 GGTTATGCAGGCGAGTATGAAGTACTTTTAAATCATGGTAGTAAGTTTAAAATTAGTAAA

440 I D S Y K D G T T T K L I V D R T L I D
1681 ATAGATTCTTATAAAGATGGTACTACAACAAAACTAATTGTTGATCGAACATTAATAGAT

* *M K N K K I*
1741 TGATTTTTAGAAAAATAATTTCTAATTCAAAGGAGGGAAAATGATGAAGAACAAAAAAA

7 *L G L L T C T V L V G Q M M T Y P V Y A*
1801 TATTAGGTCTTTTGACATGTACAGTTTAGTTGGACAAATGATGACATATCCTGTATATG

27 *K T I T Q N Y D N Q E V E T T N E K* T V
1861 CAAAGACTATTACGCAAAATTATGATAATCAGGAAGTAGAAACAACCAATGAAAAGACAG

47 S S N G L M G Y Y F A D E H F K D L E L
1921 TATCTAGTAATGGATTAATGGGTTATTATTTTGCTGATGAACATTTTAAAGATTTAGAAT

67 M A P V K N G E L K F E K N K V E K L L
1981 TAATGGCACCAGTTAAAAATGGTGAATTAAAATTTGAAAAAAATAAAGTAGAAAAGCTTT

87 T E E K T N I K S I R W T G R I I P S K
2041 TAACAGAAGAAAAAACAAATATAAAATCCATTCGTTGGACAGGAAGAATAATTCCTTCAA

107 D G E Y T L S T D K D N V L M Q I N A E
2101 AGGATGGTGAATATACATTATCAACTGATAAAGATAATGTATTGATGCAAATAAATGCTG

127 G E I A N T L K V N M I K G Q E Y S I R
2161 AAGGTGAAATTGCTAATACACTTAAAGTTAATATGATTAAAGGTCAGGAGTACAGTATCA

147 I E I Q D K D I G Y V D D L S S P K L Y
2221 GGATAGAAATACAAGATAAAGATATAGGATATGTTGATGATCTATCATCCCCTAAACTTT

167 W E L N G D K T L I P E K N L F L R D Y
2281 ATTGGGAATTAAATGGCGATAAAACACTTATTCCCGAAAAAAACTTATTCTTGAGAGATT

187 S K I D E N D P F I P K D N F F D L K L
2341 ACTCTAAAATAGATGAAAATGATCCGTTTATACCTAAAGATAACTTCTTTGATCTAAAAT

207 K S R S A R L A S G W G D E D L D T D N
2401 TAAAATCAAGATCAGCAAGACTTGCATCTGGCTGGGGAGATGAAGATTTAGATACTGATA

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227 D N I P D A Y E K N G Y T I K D S I A V
2461 ATGATAATATTCCTGATGCCTATGAAAAAAATGGTTATACTATTAAAGATTCAATTGCAG

247 K W E D S F A Q Q G Y K K Y L S S Y L E
2521 TAAAATGGGAAGATAGTTTTGCCCAGCAAGGATATAAAAAGTATTTATCAAGTTATTTAG

267 S N T A G D P Y T D Y Q K A S G S F D K
2581 AATCAAATACTGCTGGAGATCCTTATACAGACTATCAAAAAGCTTCTGGCTCTTTTGATA

287 A I K A E A R D P L V A A Y P V V G V G
2641 AAGCTATAAAAGCTGAAGCAAGAGATCCTTTAGTTGCTGCGTATCCAGTTGTAGGAGTCG

307 M E K L I I S T N E H A S T D Q G K T V
2701 GAATGGAAAAATTAATTATATCTACTAATGAACATGCATCAACTGATCAGGGCAAGACAG

327 S R N T T N S K T D A N T A G V A I N I
2761 TTTCAAGAAATACTACAAATAGTAAAACTGATGCAAATACAGCTGGAGTAGCAATTAATA

347 A Y Q N G F T G S I T T N Y S H T T E N
2821 TTGCATATCAAAATGGATTTACTGGCAGTATAACTACAAATTATTCTCATACTACAGAAA

367 S T A V Q N S N G E S W N T S L S I N K
2881 ATTCAACTGCGGTACAAAATAGTAATGGAGAATCATGGAATACTTCATTAAGTATAAATA

387 G E S A Y I N A N V R Y Y N T G T A P M
2941 AAGGTGAATCAGCATATATTAATGCAAATGTTAGATATTATAATACTGGTACTGCACCTA

407 Y K V T P T T N L V L D G D T L T T I K
3001 TGTATAAAGTAACACCGACAACTAATTTAGTATTAGATGGAGATACATTAACAACTATAA

427 A Q D N Q I G N N L S P N E T Y P K K G
3061 AAGCACAAGATAATCAAATTGGTAATAACTTATCTCCAAATGAAACATATCCTAAAAAAG

447 L S P L A L N T M D Q F S S R L I P I N
3121 GATTATCCCCTTTAGCACTTAATACAATGGATCAATTTAGTTCTAGATTAATTCCAATAA

467 Y D Q L K K L D A G K Q I K L E T T Q V
3181 ACTATGATCAATTAAAAAAATTAGATGCTGGAAAACAAATTAAACTAGAAACAACTCAAG

487 S G N Y G I K N S Q G Q I I T E G N S W
3241 TAAGTGGAAATTATGGAATTAAAAATAGTCAGGGTCAAATAATTACAGAAGGAAACAGCT

507 S D Y I S Q I D S L S A S I I L D T G S
3301 GGTCTGATTATATCAGTCAAATTGATAGCCTTTCTGCATCTATTATATTAGATACAGGCA

527 D V F E R R V T A K D S S N P E D K T P
3361 GTGATGTGTTTGAAAGACGAGTTACTGCTAAGGATTCTAGTAATCCAGAAGATAAAACAC

547 V L T I G E A I E K A F G A T K N G E I
3421 CAGTACTTACAATTGGAGAGGCAATTGAAAAAGCTTTTGGTGCTACTAAAAACGGCGAAA

567 L Y F N G M P I D E S C V E L I F D G N
3481 TATTATATTTTAATGGTATGCCAATTGATGAAAGTTGTGTTGAACTTATATTTGATGGTA

587 T A N L I K E R L N A L N D K K I Y N V
3541 ATACAGCTAACTTAATTAAAGAGCGTTTAAATGCATTAAATGATAAAAAGATATATAATG

Fig. 2/3

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 607   Q  L  E  R  G  M  K  I  L  I  K  T  S  T  Y  F  N  N  F  D
3601  TTCAACTTGAAAGAGGAATGAAGATTCTTATAAAAACATCTACATATTTTAATAATTTTG

 627   G  Y  N  N  F  P  S  S  W  S  N  V  D  S  N  N  Q  D  G  L
3661  ATGGATATAATAATTTTCCTAGTTCATGGAGTAATGTTGACTCTAACAATCAAGATGGAT

 647   Q  N  A  A  N  K  L  S  G  E  T  K  I  V  I  P  M  S  K  L
3721  TGCAAAATGCAGCAAATAAATTAAGTGGAGAGACAAAGATTGTAATACCTATGTCTAAAT

 667   N  P  Y  K  R  Y  V  F  S  G  Y  L  K  N  S  S  T  S  N  P
3781  TAAATCCATATAAACGTTATGTTTTTAGTGGATATTTGAAAAACTCTTCTACTTCTAATC

 687   I  T  V  N  I  K  A  K  E  Q  K  T  Y  N  L  V  S  E  N  D
3841  CAATTACAGTAAATATTAAAGCTAAAGAACAAAAGACATATAATTTAGTGTCAGAGAATG

 707   Y  K  K  F  S  Y  E  F  E  T  I  G  R  D  A  S  N  I  E  I
3901  ATTATAAAAAATTTAGTTATGAATTTGAGACAATTGGAAGAGATGCTTCTAATATAGAAA

 727   T  L  T  S  S  G  T  I  F  L  D  N  L  S  I  T  E  L  N  S
3961  TAACATTAACTAGTAGTGGTACAATATTTTTAGATAACTTATCTATTACAGAATTAAATA

 747   T  P  E  I  L  K  E  P  D  I  K  V  P  S  D  Q  E  I  I  D
4021  GTACTCCTGAAATATTAAAAGAACCAGATATCAAAGTTCCAAGTGATCAGGAAATAATAG

 767   A  H  K  K  Y  Y  A  D  L  S  F  N  Q  S  T  A  N  Y  Y  L
4081  ATGCACATAAAAAATATTATGCAGATTTAAGCTTTAATCAAAGTACAGCAAATTATTATT

 787   D  G  L  Y  F  E  P  T  Q  T  N  K  E  V  L  D  Y  I  Q  K
4141  TAGATGGTTTATATTTTGAACCAACTCAAACTAATAAAGAAGTACTTGATTATATCCAAA

 807   Y  K  V  E  A  T  L  E  Y  S  G  F  K  D  I  G  T  K  D  K
4201  AGTATAAAGTTGAAGCTACTTTAGAATATTCTGGATTTAAGGATATTGGAACTAAGGATA

 827   E  L  R  N  Y  T  G  D  S  N  Q  P  K  T  N  Y  V  N  F  R
4261  AGGAACTTCGTAATTATACAGGAGATTCTAATCAGCCTAAAACTAATTATGTTAATTTTA

 847   S  Y  F  T  S  G  E  N  V  M  P  Y  K  K  L  R  I  Y  A  I
4321  GAAGTTATTTTACAAGTGGAGAGAATGTCATGCCATATAAAAAATTAAGAATATATGCAA

 867   T  P  E  N  K  E  L  L  V  L  S  I  N  *
4381  TTACTCCAGAGAATAAAGAATTATTAGTACTTAGCATTAATTAGTGTTAAGGATATGTAA
4441  ATTATTAAATTTATATCTAAGATGA  4465
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Fig. 2/4

tide (19 kDa). The N-terminal part of mature Ib has a hydrophobic sequence (Leu-293 to Ser-309) predicted to form a transmembrane segment which could be involved in the translocation of the toxin across the cell membrane (PERELLE et al., 1993). There are similar segments in Sb (Leu-296 to Ser-313) (Fig. 2) and in CDTb (Leu-293 to ser-310). A conserved ATP/GTP binding site is localized 5 nucleotides downstream from the hydrophobic segment of Sb (Fig. 2), Ib and CDTb (PERELLE et al., 1993; PERELLE et al., 1997). Thus the domain organizations of C. *spiroforme* toxin, iota toxin and CDT are presumably similar.

Mapping iota toxin and iota-like toxin genes in *C. perfringens*, *C. spiroforme* and *C. difficile* strains

A series of 7 strains of *C. perfringens*, *C. difficile* and *C. spiroforme* producing iota or iota-like toxins were selected to study the genomic localization of the iota toxin genes. *C. perfringens* type D strain 945P which contains a 120 kb plasmid harboring the enterotoxin gene *(cpe)* and epsilon toxin gene *(etx)* (CORNILLOT et al., 1995) was used as a control. The *iap* and *ibp* gene probes hybridized with a large plasmid DNA from each of the 4 C. *perfringens* E strains, indicating that the iota toxin genes

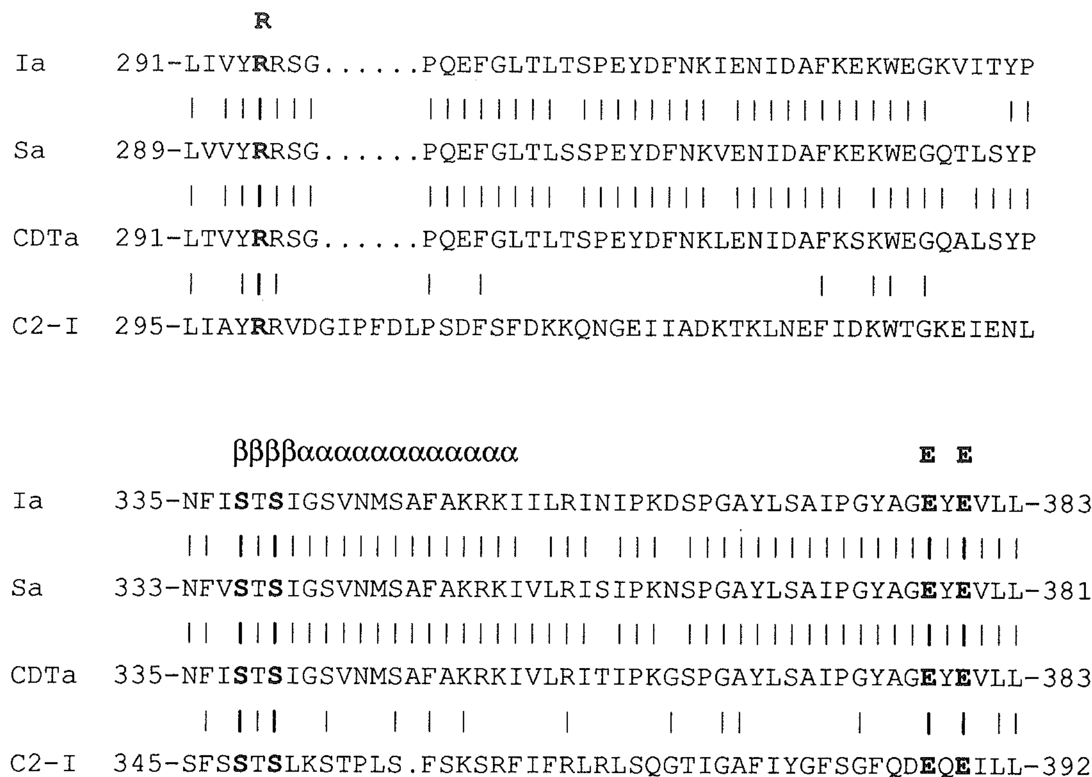

Fig. 3. Sequence alignment of the ADP-ribosylation site of Sa, Ia and CDTa. α, α-helix; β, β-strand. R and E indicates the conserved catalytic residues. The amino acids shown in boldface correspond to the conserved residues of the ADP-ribosyltransferase toxins (DOMENIGHINI and RAPPUOLI 1996).

are located on plasmid DNA in *C. perfringens* type E. The plasmids from *C. perfringens* E purified on CsCl gradients varied in size from approximately 120 to 140 kb. The plasmids also had different *Eco*RI restriction patterns (Fig. 4 and data not shown) indicating that they are not all identical, except those of NCIB10748 and NCTC8084. Strain NCIB10748 also harbored additional small plasmids (Fig. 4).

The plasmids of *C. perfringens* NCIB10748 and NCTC8084 digested with *Eco*RI showed similar hybridization patterns with *iap* and *ibp* probes (data not shown). An *Eco*RI site was present in the coding region of Ib. This suggests that these strains harbor identical plasmids. In contrast, plasmids from strains NCIB10748 and CN5065 (kindly provided by K. AKTORIES) had different hybridization patterns with *iap* and *ibp* probes (Fig. 4), suggesting that these strains have different toxin gene sequences. Partial DNA sequencing (500 nucleotides) of the *iap* gene from strain CN5065 showed a slightly different sequence (80% amino acid identity) to that of strain NCIB10748 (data not shown). The lack of the *ibp* gene in strain 46088 (Fig. 5) was also confirmed by the absence of production of Ib. Culture supernatant did not induce an iota cytotoxic effect on Vero cells but was able to ADP-ribosylate cellular actin *in vitro* (data not shown), indicating that strain 46088 only produces Ia component.

The *C. spiroforme* strains tested CS246, and NCTC11493, and the *C. difficile* strain CD196 also harbor large plasmids of similar size to the *C. perfringens* E plasmids (data not shown). However, in these strains the *iap* and *ibp* probes hybridized only with total DNA and not with purified plasmid DNA (Fig. 5). Both *C. spiroforme* strains showed an identical hybridization pattern, suggesting that these strains have similar coding sequences for the iota-like toxin genes, and a similar chromosomal localization. In contrast, the *iap* and *ibp* probes hybridized with larger *Eco*RI DNA fragments from *C. difficile* CD196.

These results summarized in Fig. 5, show that the iota toxin genes are plasmid borne in *C. perfringens* and that the iota-like toxin genes are localized on the chromosome in the *C. spiroforme* and *C. difficile* strains tested.

Discussion

The genetic analysis of *C. spiroforme* toxin from strain CS246 shows that the toxin components Sa and Sb are encoded by two genes arranged in the same orientation as the *C. perfringens* iota toxin genes and the *C. difficile* CDT genes (PERELLE et al., 1993; PERELLE et al., 1997). The *sas* gene is located upstream of *sbs*. Both genes are transcribed in the same orientation and are separated by a short non coding sequence (41 bp) similar to

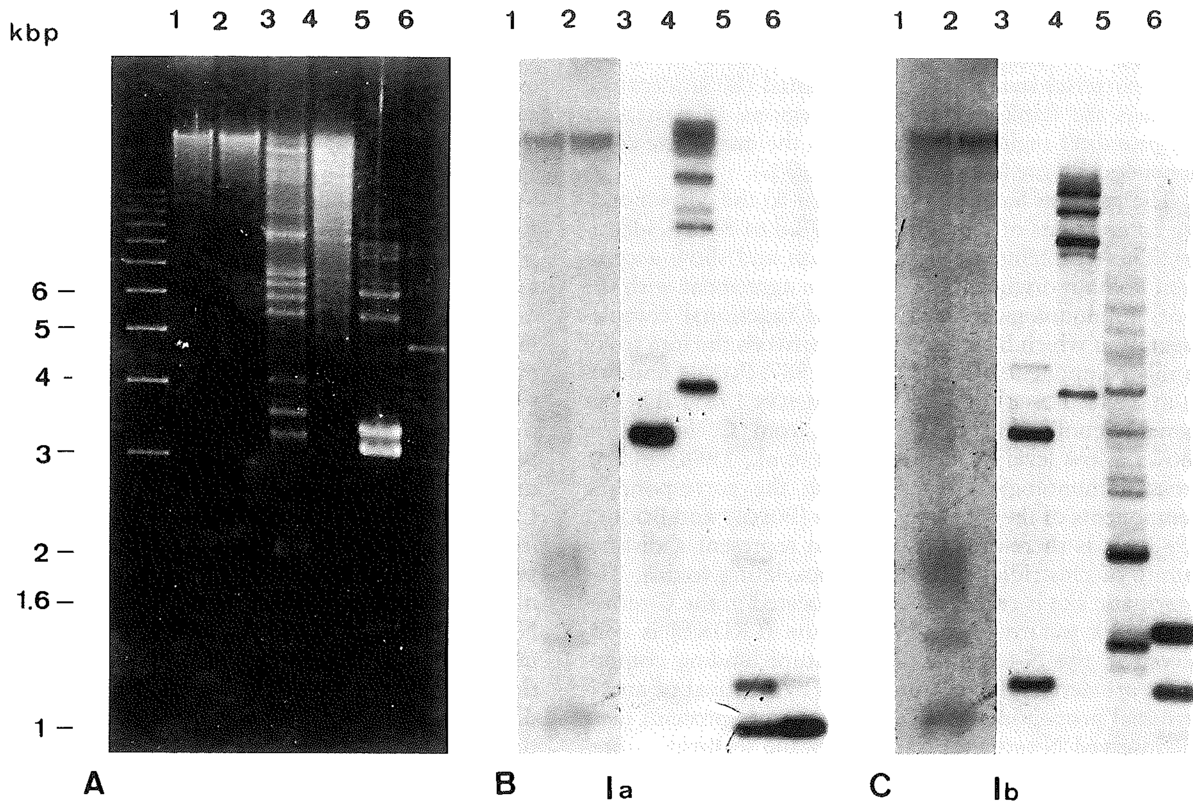

Fig. 4. (A) Agarose gel electrophoresis of plasmid DNA: NCIB10748 uncut DNA (lane 1), CN5065 uncut (lane 2), NCIB10748 *Eco*RI digested (lane 3), CN5065 *Eco*RI digested (lane 4), NCIB10748 *Hin*dIII digested (lane 5), CN5065 *Hin*dIII digested (lane 6), and (B) and (C) Southern blot with *iap* (Ia) and *ibp* (Ib) probes.

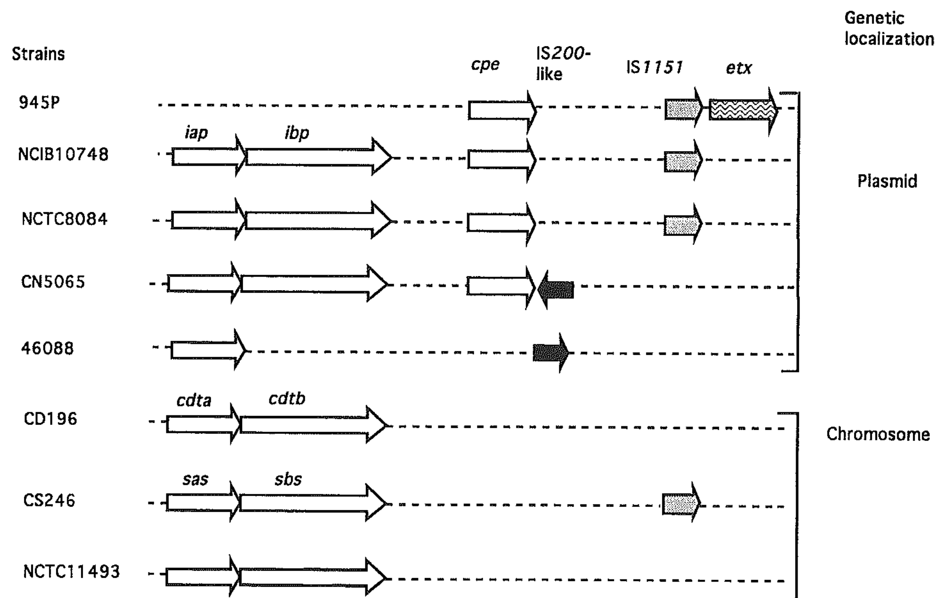

Fig. 5. Diagram showing the genomic localization of toxin genes and *C. perfringens* insertion sequences (IS*1151* and IS*200*-like element) (DAUBE et al., 1993; BRYNESTAD et al., 1994; CORNILLOT et al., 1995) in 7 *Clostridium* strains as determined by Southern blotting with gene probes reported in Table 1. The results of strain 945P are from (DAUBE et al., 1993; CORNILLOT et al., 1995).

that found in C. *perfringens* (40 bp), and in C. *difficile* (52 bp). The coding sequences of the toxins from the three bacterial strains are highly related (80% identity), whereas the flanking non coding regions, in particular the promoter region upstream from the enzymatic component genes, are less similar (44 to 60%).

The proteins Sa and Sb are similar to the corresponding components of iota and CDT (78.8 to 84.2% identity). These proteins are of similar size and show a common domain organization. This is in agreement with the cross immunological reactions and functional complementation which have been found between the iota toxin family components (EKLUND et al., 1987; POPOFF and BOQUET 1988; POPOFF et al., 1988; SIMPSON 1989). The enzymatic component of C2 toxin from C. *botulinum* shows a low level of identity (FUJII et al., 1996) and no cross immunological reaction with the corresponding components of the iota toxin family (POPOFF and BOQUET 1988). It is therefore considered to represent a distinct family of clostridial actin-ADP-ribosylating toxins. The enzymatic site is conserved and is located in the C-terminus and a putative actin-binding site (LKDKE) is conserved in the N-terminus. The mature binding components are derived from a precursor by removal of an N-terminal propeptide (19 kDa). The mature proteins have a conserved N-terminal transmembrane segment as predicted, which may be involved in translocation as observed with other toxins (PERELLE et al., 1993). The replacement of hydrophobic amino acids (Ala-294 and Pro-297) by charged residue (Glu) yielded unstable recombinant Ib proteins possibly by changing the conformation thereby facilitating proteolytic cleavage at sites that would be otherwise concealed. A consensus ATP/GTP binding sequence is localized downstream from the hydrophobic region in Ib, Sb and CDTb and seems to be non functional at least non essential for Ib activity, since the Ib mutant (Lys-320-Ala) is as active as the native Ib form (data not shown).

Our findings show that the iota toxin, one of the major toxins produced by C. *perfringens*, is encoded by plasmid genes. The iota toxin genes have also been found to be plasmid-borne in a different C. *perfringens* strain (KATAYAMA et al., 1996). The genes of the other major toxins (beta and epsilon) contributing to the toxinotype of C. *perfringens* are also present on large plasmids (ROKOS et al., 1978; CENARD et al., 1992). The alpha-toxin *(plc)* and perfringolysin genes which are found in almost all C. *perfringens* strains, are present on the chromosome (CANARD et al., 1992), whereas the *cpe* gene, present in a restricted number of strains (about 6%), is either chromosome or plasmid borne (CORNILLOT et al., 1995). Interestingly, the iota toxin gene sequences are not identical. Among the 4 C. *perfringens* E strains tested, 3 different *Eco*RI hybridization patterns were observed with *iap* and *ibp* probes. The partial sequencing of the *iap* gene from the strain CN5065 showed a variant sequence from that of strain NCIB10748. In contrast, the other toxin genes *(plc, cpe,* and *etx)* are very highly conserved in the different strains tested (VAN DAMME-JONGSTEIN et al., 1989; HUNTER et al., 1992, TSUTSUI, 1995 #204; CZECZULIN et al., 1993; BRYNESTAD et al., 1994; CORNILLOT et al., 1995). An interesting finding is that one C. *perfringens* strain (46088) had only the *iap* gene and produced an incomplete iota toxin consisting in only the enzymatic component.

The same degree of variation in the gene sequence of iota toxin is found between C. *perfringens,* C. *spiroforme,* and C. *difficile* (about 80% identity). The *sas* and *sbs* genes are identical in the two C. *spiroforme* strains tested according to the hybridization patterns. The highest level of identity was found between the regions coding for the mature proteins, whereas the sequences coding for the signal peptides and the flanking non coding regions are distantly related. This raises the question of the origin of the iota toxin genes and their transfer between *Clostridium* strains. The iota toxin genes on plasmids may have been transferred by conjugation between C. *perfringens* strains. Conjugation and mobilization of large plasmids in C. *perfringens* have already been reported (BREFORT et al., 1977; YOUNG et al., 1989), and may explain why C. *perfringens* strains NCIB10748 and NCTC8084 contain apparently an identical large plasmid harboring the *iap*, *ibp*, *cpe* and IS*1151*. However, the other two C. *perfringens* E strains analyzed contain different plasmids and in C. *spiroforme* and C. *difficile*, the iota-like toxin genes are located on the chromosome, although these strains also contain large plasmids. The interspecies transfer of the iota toxin genes could result from plasmid conjugation, and their transfer between plasmid and chromosome could be mediated by insertion sequences. However, IS*1151* and the IS*200*-like element which are linked to the *cpe* and *etx* genes (DAUBE et al., 1993; BRYNESTAD et al., 1994), are not directly associated with the iota toxin genes in C. *perfringens* E strains, and IS*1151* was identified in only one strain of C. *spiroforme* (Fig. 5). But, other unindentified insertion sequences or transposons could be involved in the dissemination of the iota toxin genes in *Clostridium* strain 46088 which showed a truncated iota toxin operon, suggests that homologous recombination and deletion could also occur in rearrangement of iota toxin genes.

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