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Cloning of *Clostridium difficile* toxin B gene and demonstration of high N-terminal homology between toxin A and B

Christoph von Eichel-Streiber, Rita Laufenberg-Feldmann, Sabine Sartingen, Jörg Schulze, and Markus Sauerborn

Institut für Medizinische Mikrobiologie, Johannes-Gutenberg-Universität, Hochhaus am Augustusplatz, W-6500 Mainz, Federal Republic of Germany

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Abstract. High titered *Clostridium sordellii* lethal toxin antiserum, cross-reactive with *C. difficile* cytotoxin B (ToxB), was used to isolate *toxB* fragments from a *C. difficile* expression library. Recombinant clones containing *toxB* fragments of the 5′ and 3′ end were isolate. A 2.5-kb *Hin*cII fragment of chromosomal DNA overlaps both groups of clones. A partial restriction map of the total *toxB* gene is presented. The gene is positioned upstream of *utxA* and *toxA*. *toxB* has a size of 6.9 kb, corresponding to a 250-kDa polypeptide. A partial sequence of the 5′ end of *toxB* was determined. The sequence contains 398 bp upstream of *toxB* with a putative Shine-Dalgarno box (AGGAGA) and 609 bp of the *toxB* open reading frame. The N-terminal 203 amino acids of ToxB were compared with the N-terminal amino acids of the enterotoxin A (ToxA). A homology of 64% of the residues was detected, which proves the relatedness of ToxA and ToxB of *C. difficile*.

Introduction

In the late seventies a toxic activity neutralizable with *Clostridium sordellii* antitoxin was detected in stool specimens from patients suffering from Pseudomenbranous Colitis (PMC) (Bartlett et al. 1978). Today it is well known that PMC is caused by pathogenic *Clostridium difficile* strains. Two toxins are produced by either *C. difficile* and *C. sordellii*. The enterotoxin A (ToxA) of *C. difficile* is related to the hemorrhagic toxin (HT) of *C. sordellii*, and the cytotoxin B (ToxB) of *C. difficile* is related to the lethal toxin (LT) of *C. sordellii*. This relatedness was discovered by the use of cross-reactive antisera (Chang et al. 1978). Neutralization of cytotoxic activity by the cross-reactive *C. sordellii* LT antiserum is routinely used for the detection of *C. difficile* ToxB in stool specimens of humans.

Offprint requests to: C. von Eichel-Streiber

Abbreviations: PBS, phosphate-buffered saline; ToxA, *C. difficile* enterotoxin A; ToxB, *C. difficile* cytotoxin B; *toxB*, gene encoding ToxB; HT, *C. sordellii* hemorrhagic toxin; LT, *C. sordellii* lethal toxin

C. difficile ToxA and ToxB are released into the supernatant of the growing cell cultures. The toxin A gene (*toxA*) has been sequenced and it is now known that ToxA is a single chain protein of 308 kDa and pI 5.3 (Dove et al. 1990; Sauerborn and Eichel-Streiber 1990). Characterization of the 250 to 500-kDa ToxB of *C. difficile* has proven to be very difficult. ToxB preparations are regularly contaminated with a 150-kDa protein that shows 50-kDa subunits after reduction (Pothoulakis et al. 1986). Special purification conditions have been elaborated to separate ToxB from this contaminant (Eichel-Streiber et al. 1987; Meador and Tweten 1988). Meador and Tweten prepared homogeneous ToxB and determined its N-terminal amino acid sequence. This was the basis for the isolation of *ToxB* fragments derived from its 5′ end (Schulze and Eichel-Streiber 1990).

The aim of the present study was to isolate ToxB-positive clones from a *C. difficile* expression library with the aid of *C. sordellii* LT antiserum. These experiments have led to the complete characterization of *ToxB*. A partial sequence of its 5′ end shows the relatedness between ToxA and ToxB.

Material and methods

Characterization of C. sordellii *lethal toxin antiserum: cross-reactivity with ToxB*

The *C. sordellii* lethal toxin antiserum (Wellcome, Beckenham, UK) was titered for ToxB and ToxA reactivity. Purified toxins (0.3 µg/20 µl per well; 2 h; room temperature) were absorbed onto enzyme-linked immunosorbent assay (ELISA) 96-well microtiter plates (Becton and Dickenson, Heidelberg, FRG). Serial dilutions of *C. sordellii* antiserum were added to the plates for 1 h at 37°C temperature. Immunoassays were developed with peroxidase-labelled rabbit anti-horse IgG (Dianova, Hamburg, FRG) and 2.2-azino-di-[3-ethyl-benzthiazolinsulfonate (6)] [10-mg/ml ABTS (Boehringer, Mannheim, FRG), 0.0025% H_2O_2] as a substrate. ELISA plates were read at 414 nm (Titertek Multiscan ELISA reader; Flow, Meckenheim, FRG). A polyclonal rabbit antiserum against ToxB that was produced in our laboratory was tested in parallel. Western blot analysis was carried out as previously described (Eichel-Streiber et al. 1987).

Recombinant DNA techniques

The construction of the genetic library has been described (Eichel-Streiber et al. 1989). In short, partial *Alu*I, *Hae*III fragments of chromosomal DNA of *C. difficile* VPI10463 were cloned into the *Sma*I site of the plasmid vector pUC12 and transformed into *E. coli* JM83 (Yanisch-Perron et al. 1985). All standard recombinant DNA techniques were performed as described by Maniatis et al. (1982). Oligodeoxyribonucleotide Boli1 (41mer) synthesized in the laboratory of Dr. Engels (Universität Frankfurt, FRG), was used to determine the position of the 5′ end of *ToxB*. It was radioactively labelled by a kinase reaction (Maniatis et al. 1982).

Colony blotting and detection of recombinant protein

The genomic library was plated at a cell density of 8,000–10,000 colonies on a 24 × 24 cm plate and cells were grown overnight. Colonies were transferred to nitrocellulose (NC) and lysis was achieved by incubation of the NC filter layered on 3 M Whatman paper soaked with 5% SDS solution (15–20 min at 90°C). Lysed cells were electroblotted (1 h, 50 V = const) to fix the protein on the membrane and reduce the SDS concentration. Filters were washed three times (was buffer: PBS 25 mM, pH 7.5, 0.5% gelatine, 0.1% Triton X-100). Staining was performed with *C. sordellii* LT antiserum (1:2,000), peroxidase-labelled rabbit anti-horse IgG (1:2,000), and diaminobenzidine (0.5 mg/ml; 0.006% H_2O_2) as substrate. All dilutions were made in wash buffer.

Recombinant protein was produced and analyzed as already described (Eichel-Streiber et al. 1989). Staining of ToxB fragments was done as described for the primary screening (see above), except that the antibody concentrations used were 1:5,000.

DNA sequencing and analysis

T7-DNA polymerase sequencing of plasmid DNA was done as described by Tabor and Richardson (1987), using a Pharmacia Sequencing Kit (Pharmacia, Freiburg, FRG). Kinased Boli1, standard or reverse primers were used (Biolabs, Schwalbach, FRG). Wedge-shaped sequencing gels (60 cm length; 0.2–0.4 mm thick; 6% (polyacrylamide and 7 M urea) were run at 3,000 V with a restriction of power to 50 W.

The DNA sequence analysis package of Guido Volkaert (Leuwen, Belgien) and PC/Gene version 6.0 Amos Bairoch (Genofit, Heidelberg, FRG) was used to analyze the nucleotide and amino acid sequences.

Results

Characterization of C. sordellii *antiserum*

The *C. sordellii* LT antiserum and a ToxB antiserum were tested in an ELISA using ToxB and ToxA as antigens (Fig. 1). The ToxB antiserum exhibited antibody titers of $1:8\times10^5$ and $1:2\times10^5$ against ToxB and ToxA, respectively. The *C. sordellii* LT antiserum exhibited a titer of $1:5\times10^6$ against ToxB and a low titer of $1:2\times10^4$ against ToxA. To confirm that the LT antiserum detected *C. difficile* ToxB polypeptide, we also used the serum in a Western blot analysis (Fig. 2). The LT

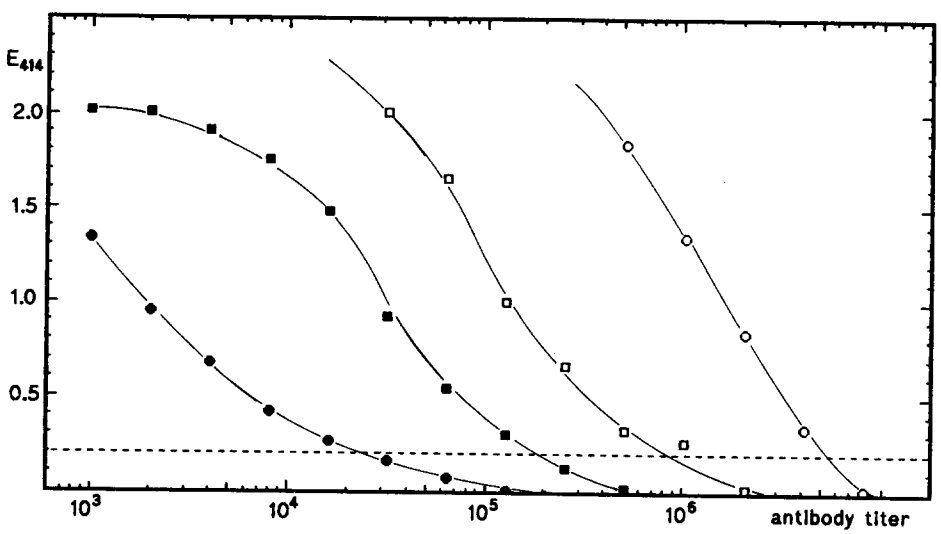

Fig. 1. Titration of antisera. *C. sordellii* lethal toxin (LT) antiserum and *C. difficile* cytotoxin B (ToxB) antiserum were titered against *C. difficile* enterotoxin A (ToxA) and ToxB of *C. difficile* in an enzyme-linked immunosorbent assay (see Material and methods). The extinction at 414 nm was measured and plotted against the respective titers. *C. difficile* ToxB antiserum against ToxB (□), ToxA (■). *C. sordellii* LT antiserum against ToxB (○), ToxA (●)

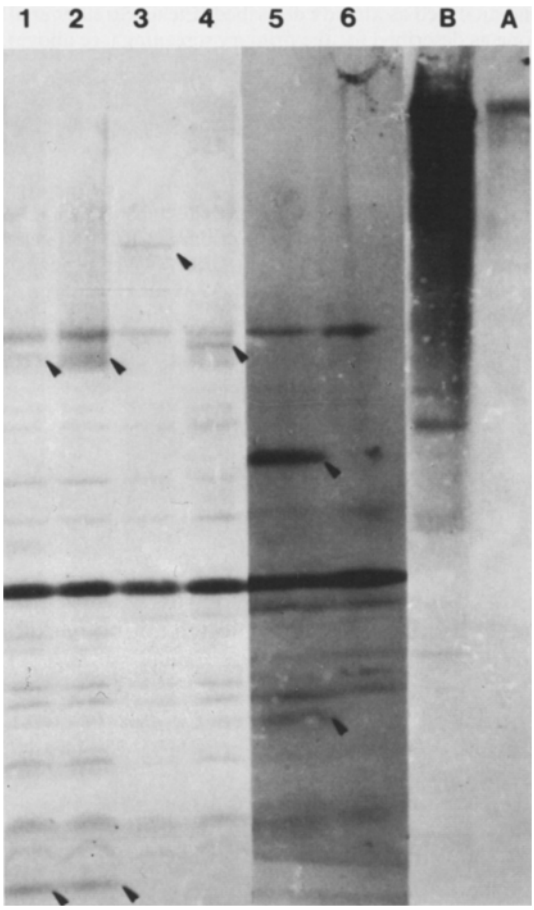

Fig. 2. Western blot of recombinant proteins. Samples of ToxB (1 μg) and total lysates of the individual recombinant clones (50 μg) were separated on a 5%–10% SDS-PAGE gel and thereafter transfered to nitocellulose. Proteins were detected using polyclonal horse serum specific for the ToxB cross-reactive *C. sordellii* LT (see results and Fig. 1). Positive control: ToxB, *Lane B*; ToxA, *lane A*. Negative control: *E. coli* JM83 transformed with pUC12, *lane 6*. Recombinant ToxB fragments of *E. coli* JM83 containing: *lane 1:* pCd122; *lane 2:* pTBs12; *lane 3:* pTBs92; *lane 4:* pTBs45; *lane 5:* pTBs72. Recombinant proteins are indicated by *arrows*

serum specifically detected the ToxB molecule within culture filtrate of *C. difficile*. A weak staining of ToxA was also detected.

Characterization of toxB

The horse LT antiserum was used for colony blotting of the gene bank of *C. difficile* VPI10463. This screening yielded 11 positive clones. Characterization of the clones was done by restriction enzyme mapping (Fig. 3) and Western blot analysis of the expressed recombinant protein (Fig. 2). Restriction digests with *Xba*I and *Eco*RV indicated a similarity of some clones to the pTB clones isolated by oligonucleotide screening (Schulze and Eichel-Streiber 1990; *Xba*I similarity); others were similar to pCd122 which encodes for the N terminus of ToxA (Eichel-Streiber et al. 1990; *Eco*RV similarity). Clones with the *Xba*I similarity hybridized with a 41mer (Boli1: 5′-ATG AGT TTA GTT AAT AGA AAA CAG TTA GAA AAA ATG GCA AAT GT-3′, data not shown) derived from the N-terminal sequence reported (Meador and Tweten 1988) and, thus, expressed parts of the N terminus of ToxB.

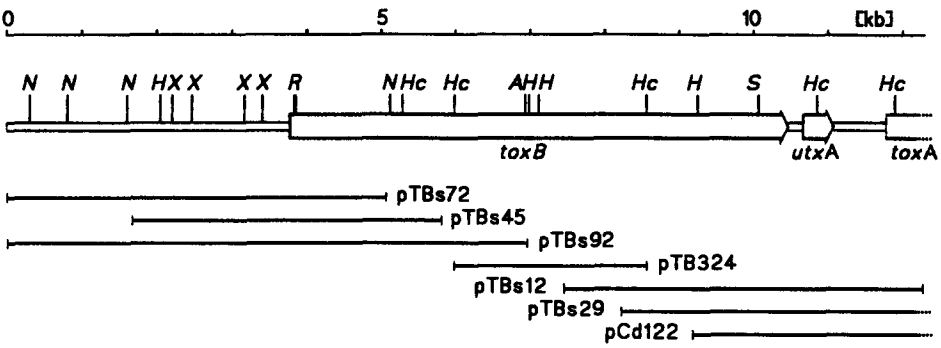

Fig. 3. Partial map of total *toxB*. Clones pTBs72, pTBs45, pTBs92, pTBs12 and pTBs29 were isolated from the colony blotting with *C. sordellii* LT antiserum. Clone pTB324 contains a *Hin*cII fragment cloned of chromosomal DNA. pCd122 encodes for the N-terminal part of ToxA. Restriction enzymes are: *A*, *Acc*I; *Hc*, *Hin*cII; *H*, *Hin*dIII; *N*, *Nde*I; *R*, *Rsa*I; *S*, *Sph*I; *X*, *Xba*I. Three open reading frames are indicated, *toxB*, *utxA*, and *toxA*

Clones with the *Xba*I similarity were used to partially sequence the 5′ end of *toxB* (Fig. 4). Constructs with the *Eco*RV-restriction pattern hybridized with pCd122 insert DNA (data not shown). No overlapping clones between these two groups were isolated from the genetic library.

Two clones pTBs92 and pTBs12 extended furthest into the *toxB* gene from the 5′ and 3′ end, respectively. Insert DNA from these clones hybridized with a 2.6-kb *Hin*cII fragment of *C. difficile* chromosomal DNA which was cloned in pTB324. Figure 3 delineates a partial map of the total *toxB* gene, whose 5′ end is located 54 bp upstream of the *Rsa*I site at 3.8 kb of the map, and the 3′ end 405 bp downstream of *Sph*I site of pCd122 (Dove et al. 1990). The gene has a size of 6.9 kb and is positioned upstream of *toxA* and *utxA* (Sauerborn and Eichel-Streiber 1990).

Expression of ToxB fragments

Western blot analysis of recombinant protein from clones of the primary screening revealed that ToxB fragments of different size were detected (Fig. 2). N-terminal ToxB fragments were expressed by clones pTBs72, pTBs45, pTBs92 and C-terminal fragments by clones pTBs12 and pCd122. A 500-bp *toxB* fragment, part of a 2.6-kb *Hin*cII fragment mentioned above, situated in the center of the gene (between 6.8 and 7.4 of the physical map, Fig. 3), could not be isolated by colony blotting.

Comparison of the N termini of ToxA and ToxB

The N-terminal 203 amino acids deduced from our partial *toxB* sequence were compared with the published ToxA sequence (Sauerborn and Eichel-Streiber 1990). Alignment of the two sequences showed a striking homology between the two toxins at their N termini (Fig. 5). Half (46%) of the deduced amino acid sequence was identical, another 18% were homologous. Some antigenic determinants were encoded by the 1,000 bp of the extreme 5′ end detected in Western blot analysis of clone pTBs72 (see Fig. 2).

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                                                                    XbaI
TCTAGACAAGCTGTTAATAAGGCTAAAAATAGAGCATTTAAAAAAATAAAAAAAGACTA      59

TGAAAATTATTTTAACTTGTAATTAATGAGCTTAAAGAGATATTTATAATAGAAATCAAA     119

TTTTAGAATTAACTTTATTGTAAAATCAATAACTTAATCTAAGAATATCTTAATTTTTAT     179

ATTTTATATAGAACAAAGTTTACATATTTATTTCAGACAACGTCTTTATTCAATCGAAGA     239

GCAAATTAATCAACTGAGTGTCTTAAATTTAAAATGTTAGGAAGTGAATGTATATGAAAA     299

CCTAAGTAGATATTAGTATATTTTATAAATAGAAAGGAGGATATATAAAAGAGTTTTAGC     359

                                                  BOLI1--TGGTTAGTAAATAGAAAA
ATTTAGATGTAAAAATATTATAGTAAAGGAGAAAATTTTATGAGTTTAGTTAATAGAAAA     419
                                                M  S  L  V  N  R  K       7

CAATTAGAAAAAATGGCAAATGT
                                                    RsaI
CAGTTAGAAAAAATGGCAAATGTAAGATTTCGTACTCAAGAAGATGAATATGTTGCAATA     479
Q  L  E  K  M  A  N  V  R  F  R  T  Q  E  D  E  Y  V  A  I      27

TTGGATGCTTTAGAAGAATATCATAATATGTCAGAGAATACTGTAGTCGAAAAATATTTA     539
 L  D  A  L  E  E  Y  H  N  M  S  E  N  T  V  V  E  K  Y  L      47

AAATTAAAAGATATAAATAGTTTAACAGATATTTATATAGATACATATAAAAAATCTGGT     599
 K  L  K  D  I  N  S  L  T  D  I  Y  I  D  T  Y  K  K  S  G      67

AGAAATAAAGCCTTAAAAAAATTTAAGGAATATCTAGTTACAGAAGTATTAGAGCTAAAG     659
 R  N  K  A  L  K  K  F  K  E  Y  L  V  T  E  V  L  E  L  K      87

AATAATAATTTAACTCCAGTTGAGAAAAATTTACATTTTGTTTGGATTGGAGGTCAAATA     719
 N  N  N  L  T  P  V  E  K  N  L  H  F  V  W  I  G  G  Q  I     107

AATGACACTGCTATTAATTATATAAATCAATGGAAAGATGTAAATAGTGATTATAATGTT     779
 N  D  T  A  I  N  Y  I  N  Q  W  K  D  V  N  S  D  Y  N  V     127

AATGTTTTTTATGATAGTAATGCATTTTTGATAAACACATTGAAAAAAACTGTAGTAGAA     839
 N  V  F  Y  D  S  N  A  F  L  I  N  T  L  K  K  T  V  V  E     147

TCAGCAATAAATGATACACTTGAATCATTTAGAGAAAACTTAAATGACCCTAGATTTGAC     899
 S  A  I  N  D  T  L  E  S  F  R  E  N  L  N  D  P  R  F  D     167

TATAATAAATTCTTCAGAAAACGTATGGAAATAATTTATGATAAACAGAAAAATTTCATA     959
 Y  N  K  F  F  R  K  R  M  E  I  I  Y  D  K  Q  K  N  F  I     187

AACTACTATAAAGCTCAAAGAGAAGAAAATCCTGAACTTATAATTGAT                1008
 N  Y  Y  K  A  Q  R  E  E  N  P  E  L  I  I  D                 203
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TOXA    - MSLISKEELIKLAY-SIRPRENEYKTILTNLDEYNKLTTNNNENKYLQLK  -49
          ***. .  * *.*    *  * ** .**  *.**  .. *    *** **
TOXB    - MSLVNRKQLEKMANVRFRTQEDEYVAILDALEEYHNMSENTVVEKYLKLK  -50

TOXA    - KLNESIDVFMNKYKTSSRNRALSNLKKDILKEVILIKNSNTSPVEKNLHF  -99
            .*   *...  ** * **.**   *  .. **. .** * .********
TOXB    - DINSLTDIYIDTYKKSGRNKALKKFKEYLVTEVLELKNNNLTPVEKNLHF -100

TOXA    - VWIGGEVSDIALEYIKQWADINAEYNIKLWYDSEAFLVNTLKKAIVESST -149
            ***** . * *. ** ** *.*..**. ..*** ***.*****..***.
TOXB    - VWIGGQINDTAINYINQWKDVNSDYNVNVFYDSNAFLINTLKKTVVESAI -150

TOXA    - TEALQLLEEEIQNPQFDNMKFYKKRMEFIYDRQKRFINYYKSQINKPTVP -199
            ..*    * .. * **  **..**** ***.** ******.*
TOXB    - NDTLESFRENLNDPRFDYNKFFRKRMEIIYDKQKNFINYYKAQREENPEL -200

TOXA    - TIDDIIKSHLVSEYNRDETVLESYRTNSLRKINSNHGIDIRANSLFTEQE -249
            **
TOXB    - IID---------------------------------------------- -203
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Fig. 5. Comparison of the ToxA and ToxB N termini. Of the 203 amino acids compared 94 (46%) are identical (*) and 37 (18%) are similar (.)

Discussion

Immunological cross-reactivity between *C. difficile* ToxB and *Clostridium sordellii* LT antisera had been reported by several groups (Chang et al. 1978; Popoff 1987). Compared to the ToxB serum, the LT serum has a higher ToxB and a lower ToxA titer. The high antibody titer against ToxB could have been due to lower toxicity of LT compared to ToxB (Gill 1982; Popoff 1987). Additionally the LT toxin may be a better immunogen or it may lack immunosuppressive effects of ToxB (Däubener et al. 1988).

Because of their relatively low titers and cross-reactivity with ToxA, ToxB antisera could not be used to screen our genetic library. With the use of the antiserum against *Clostridium sordellii* LT, however, recombinant clones situated at the *toxB* 5′ and 3′ end were identified and isolated. A *Hin*cII fragment, detected by Southern analysis of chromosomal DNA, joins and overlaps the two parts of *toxB* (Fig. 3, pTB324). The *toxB* genetic map is similar to the map presented by Johnson et al. (1990). *toxB* and *toxA* are encoded close to each other. A third open reading frame designated *utxA* whose function is unknown, is positioned between

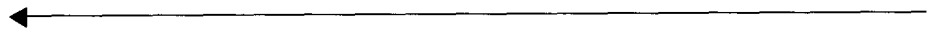

Fig. 4. DNA sequence of the 5′ end of *toxB*. The listed partial DNA sequence starts with the *Xba*I site at 3.5 kb of the physical map (see Fig. 3) and contains 398 bp upstream of *toxB* and 609 bp of the coding region. The two asymmetrical restriction sites *Xba*I and *Rsa*I are indicated. The BoliI sequence is written on top of the DNA sequence determined. Boxed amino acids are identical to those reported by Meador and Tweten (1988) for the N terminus of ToxB, except the Ser

these genes. The size of the cloned *C. difficile* insert DNA might have permitted isolation of overlapping clones encoding the total *toxB* gene. Why there was a gap positioned in the center of the *toxB* gene between the two groups of clones from the primary screening is not understood yet.

Johnson et al. (1990) cloned a *Xba*I-*Sph*I fragment of chromosomal DNA and reported that *E. coli* clones expressing active ToxB were stable. Thus, production of a possible "suicide fragment" derived from the central part of ToxB can be ruled out. None of the clones isolated by us exhibited cytotoxic activity (data not shown). In contrast to recombinant clones expressing ToxA fragments of the immunodominant C-terminal third of ToxA (Eichel-Streiber et al. 1989), the isolated ToxB fragments resided at the 5′ and 3′ end of the *toxB* gene.

Determination of the 5′-sequence of *toxB* revealed identity of the deduced amino acid sequence with the published N-terminal sequence of ToxB with one difference, i.e., the N-terminal Ser which had been reported to be a Trp (Meador and Tweten 1988). Comparison of the N termini of ToxA and ToxB revealed a marked homology of about 64% of the amino acids analyzed (Fig. 5). This homology had already been predicted from the reactivity of some monoclonal antibodies, which detect both toxins in ELISA and Western blot analysis (Eichel-Streiber et al. 1987; Rothman et al. 1988). DNA sequencing thus confirms earlier findings that ToxA and ToxB are related molecules (Eichel-Streiber et al. 1987). Although Lyerly et al. (1989) have reported that certain antibodies directed against unrelated proteins may interact unspecifically with both toxins, the presented sequence comparison indicates that this general contention can be ruled out for the described monoclonal antibodies.

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