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Nucleotide Sequence of the Streptococcal Pyrogenic Exotoxin Type B Gene and Relationship between the Toxin and the Streptococcal Proteinase Precursor

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The streptococcal pyrogenic exotoxin (SPE) type B-encoding structural gene, *speB*, was subcloned from a 4.5-kilobase streptococcal DNA insert onto a 2.4-kilobase insert, which was then sequenced. Studies indicated that a 1,194-base-pair open reading frame encoded a 398-amino-acid protein. Removal of the putative signal peptide resulted in a mature protein with 371 residues (molecular weight, 40,314), which was subsequently proteolyzed to yield a 253-residue breakdown product (molecular weight, 27,588). This processing was confirmed by amino-terminal sequencing of both the 40,314-molecular-weight protein and the breakdown product. Monte Carlo analysis indicated that SPE B was relatively dissimilar to other members of the pyrogenic toxin family that also includes SPEs A and C, toxic shock syndrome toxin 1, and the staphylococcal enterotoxins. Comparison with the published amino acid sequence of streptococcal proteinase precursor as well as DNA hybridization experiments indicated that SPE B is a variant of this protein even though the particular gene sequenced did not encode a proteolytically active molecule.

Streptococcal pyrogenic exotoxin type B (SPE B) is a member of the family of pyrogenic toxins (PTs), which share biological and biochemical properties (7, 39, 43). Other members include staphylococcal enterotoxin serotypes A to E (SEA to SEE), staphylococcal pyrogenic exotoxins A and B, toxic shock syndrome toxin 1 (TSST-1), and streptococcal pyrogenic exotoxin types A and C (SPE A and SPE C, respectively). Properties characteristic of this family of toxins are T-cell mitogenicity, the ability to cause a scarlet fever-like rash, pyrogenicity, immunosuppression, and the ability to enhance susceptibility to endotoxin shock (7, 39, 43). Streptococcal pyrogenic exotoxins have the additional ability to cause myocardial necrosis (32).

The nucleotide sequences of the genes encoding SPE A, SPE C, TSST-1, and SEA, -B, -C1, -C2, -D, and -E are known (3, 4, 6, 9, 10, 13, 21, 23, 45). Comparisons of amino acid sequences have shown that most of these toxins are highly similar but that TSST-1, despite shared biological activities, has little similarity with the other members of the group (21). All of these toxins have molecular weights of 20,000 to 30,000 and have no demonstrated enzymatic activity.

Estimates of the molecular weight of SPE B have varied from 17,000 to 30,000 (2, 8, 14, 20, 44), and the protein migrates as three bands upon isoelectric focusing, with isoelectric points of 8.0, 8.3, and 9.0 (2). Gerlach et al. (19), in subsequent studies, proposed that SPE B is identical to streptococcal proteinase precursor (SPP), an extracellular zymogen elaborated by group A streptococci (16, 17). This precursor has a reported molecular weight of 36,708 and undergoes autocatalysis upon reduction to yield a 28,000-molecular-weight active proteinase (47). The identity of SPE B and SPP proposed by Gerlach et al. would dictate that SPE B have this higher molecular weight and that autocatalysis lead to proteolysis products with smaller sizes and altered charge properties.

This study was undertaken to determine accurately the molecular weight of SPE B and to ascertain its relationship to the other PTs and to SPP.

MATERIALS AND METHODS

Bacterial strains and growth conditions. SPE B was purified from cultures of *Streptococcus pyogenes* 86-858 (M type 12) (8) or NY-5 (M types 10 and 12) (2). These strains, along with *S. pyogenes* C203S (M type 3) (41), C203U (M type 3) (33), and T19 (M type 19) (2) were also used in DNA hybridization experiments. Plasmids were propogated in *Escherichia coli* JM109 [*recA1* Δ(*lac pro*) *endA1 gyrA96 thi-1 hsd17 supE44 relA1* F′ *traD36 proAB*$^+$ *lacI*^{q} ZΔM15] or K-12 RR1 (F$^-$ *hsdS20 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$). Bacteriophage M13 clones were propogated in *E. coli* JM101 (Δ(*lac-pro*) *supE thi* F′ *traD36 proAB lacI*^{q} ZΔM15 [11]) under standard conditions (28).

Toxin purification. SPE B was purified in one of two ways. A standard purification protocol was described by Wannamaker and Schlievert (43). Briefly, streptococci were grown in dialyzable beef heart medium. Cultures were treated with ethanol and toxin resolubilized in acetate-buffered saline, dialyzed against distilled water, and lyophilized. The resulting material was digested with hyaluronidase and subjected to repeated preparative thin-layer isoelectric focusing. The appropriate fractions were collected, dialyzed for 4 days against pyrogen-free water to remove ampholytes, and again lyophilized.

In an effort to prevent possible proteolysis, a second method was developed and also used to purify SPE B. Streptococcal cultures were treated with ethanol and toxin resolubilized in water and dialyzed against 0.12 M sodium iodoacetate (Sigma Chemical Co., St. Louis, Mo.), a proteinase inhibitor, to prevent proteolysis. Subsequent prepar-

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ative thin-layer isoelectric focusing was performed in a pH gradient of 7.0 to 9.0. The appropriate fractions were dialyzed against 0.06 M sodium iodoacetate and then pyrogen-free water and were lyophilized. This above protocol will be referred to as the proteinase inhibitor method of SPE B purification.

Amino acid sequencing. The amino-terminal amino acid sequences of SPE B purified by the standard protocol as well as that prepared by the proteinase inhibitor protocol were determined by automated amino-terminal peptide sequencing (model 470A gas-phase protein sequencer; Applied Biosystems, Foster City, Calif.). This system employs the automated Edman degradation technique of Hewick et al. (22). SPE B purified by the proteinase inhibitor protocol was subjected to high-pressure liquid chromatography (online model 120A; Applied Biosystems) before sequencing.

Subcloning experiments. The gene encoding SPE B, designated *speB*, was previously shown (8) to be present on a 4.5-kilobase (kb) *Bam*HI-*Bgl*II streptococcal DNA insert in pUC18 (46). This construct, designated pUMN713, codes for a protein identical to streptococcus-derived SPE B (8). Repeated efforts to further subclone *speB* by random restriction enzyme digestions and subsequent fragment religation into pUC18 were unsuccessful, and an alternate strategy was adopted. The amino-terminal sequence of SPE B purified by the standard protocol was determined. This information allowed the synthesis of a complementary oligonucleotide. The oligonucleotide was 3′ end labeled (12) with [α-^{32}P]dATP (Dupont, NEN Research Products, Boston, Mass.) and used as a probe in subsequent hybridizations. The 4.5-kb insert of pUMN713, which contains *speB*, was digested with *Hin*dIII, *Kpn*I, *Dra*I, *Cla*I, or *Pvu*II. The resulting fragments were electrophoresed in an agarose gel, transferred to nitrocellulose (37), and hybridized with labeled complementary oligonucleotides (34).

These hybridization studies indicated that *speB* was contained on a 2.4-kb *Bam*HI-*Kpn*I fragment of pUMN713. This fragment was isolated, ligated into pUC18 (yielding a construct designated pUMN720), and transformed into *E. coli* JM109. Transformants were positive for SPE B production, as assayed by Ouchterlony immunodiffusion with hyperimmune SPE B rabbit antisera (29).

M13 cloning. Specific fragments of DNA were prepared for sequencing by one of three methods. (i) The 2.4-kb *speB*-containing insert was removed from pUMN720 by digestion with *Eco*RI and *Xba*I and ligated into doubly digested M13 replicative-form DNA. The resulting constructs were transformed into *E. coli* JM101 by the method of Messing (28), and recombinants were screened for SPE B production by Ouchterlony immunodiffusion with hyperimmune SPE B rabbit antisera. Single-stranded phage were isolated from SPE B-producing plaques. Deletion subclones were then made (15). (ii) Small subfragments of the 2.4-kb *Bam*HI-*Kpn*I insert were isolated by restriction endonuclease digestion, agarose gel electrophoresis, and subsequent extraction from the gel, using the rapid freezing method of Smith (35). These fragments were ligated into appropriately digested M13 vectors, and the resulting constructs were transformed into *E. coli* JM101. (iii) A 17-nucleotide fragment was synthesized by the Microchemical Facility of the University of Minnesota. This oligonucleotide was chosen such that it could act as a specific primer in subsequent sequencing experiments in which it was allowed to hybridize to *speB*-containing M13 templates.

In all cases, single-stranded templates for sequencing were prepared as described by Messing (28) and sequenced by the dideoxy method of Sanger et al. (31). All reagents were obtained from United States Biochemical Corp. (Cleveland, Ohio) or Dupont and used as instructed by the manufacturers.

Computer analysis. All computer analyses were performed by using the Molecular Biology Information Resource (Department of Cell Biology, Baylor College of Medicine, Houston Tex.). Monte Carlo analysis was performed by using the SS2 alignment algorithm of Altschul and Erickson (1). A protein sequence search of the Protein Information Resource (version 20) sequence bank was performed.

SDS-polyacrylamide gel electrophoresis and Western immunoblot assays. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by using the discontinuous buffer system of Laemmli (24). Protein bands were visualized by staining with Coomassie brilliant blue R250. Western immunoblots were performed by first transferring proteins from polyacrylamide gels to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) in a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.) filled with transfer buffer (25 mM Tris buffer [pH 8.3], 192 mM glycine, and 20% [vol/vol] methanol). Nitrocellulose membranes were incubated at room temperature in the presence of diluted hyperimmune rabbit antiserum against SPE B, followed by incubation in alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma). The indolyl phosphate-nitroblue indicator system of Blake et al. (5) was used to detect antigen-antibody complexes.

Proteinase assay. Proteinase activity was assayed by using fluorescein isothiocyanate (FITC)-labeled casein as a substrate (42). The assay was performed under conditions similar to those used to examine the proteolytic activity of streptococcal proteinase precursor (18, 26). SPE B (50 ng) was purified from *E. coli* K-12 RR1(pUMN720), *S. pyogenes* 86-858, or *S. pyogenes* NY-5 by use of the standard protocol and was incubated for 30 min at 40°C in 0.01 mM dithiothreitol and assay buffer (0.1 M phosphate buffer [pH 7.6], 0.01 mM EDTA). This solution was added to 20 μl of 0.5% (wt/vol) FITC-labeled casein (Sigma) in assay buffer and incubated at 40°C. After 30 min or 6 h, the reaction was stopped by addition of 120 μl of 5% trichloroacetic acid, and the mixture was stored overnight at 4°C. The insoluble protein was sedimented by a 5-min centrifugation in a microfuge. A 60-μl sample of the supernatant fluid was diluted to 2.5 ml with 500 mM Tris buffer (pH 8.5). A Perkin-Elmer fluorometer (model LS5B) was used to measure fluorescence relative to that of an identical sample to which trichloroacetic acid was added at time zero. An excitation wavelength of 490 nm and an emission wavelength of 525 nm were used.

The proteolytic activity of trypsin was assayed in the same manner except that the enzyme was not reduced before use, the assay buffer was 50 mM Tris buffer (pH 7.8) containing 10 mM calcium chloride, and the incubation temperature was 37°C.

DNA hybridizations. The 3.2-kb *Eco*RI-*Dra*I fragment of pUMN713 was ligated with pBR328 (36) previously digested with *Eco*RI and *Bal*I and was cloned into *E. coli* RR1. This plasmid was designated pUMN717. A 612-base-pair (bp) internal *speB* probe was obtained as follows: pUMN717 DNA was digested with *Cla*I and *Kpn*I, and the resulting fragments were separated by agarose gel electrophoresis. The 612-bp fragment, which is an internal portion of *speB*, was extracted from the gel (35) and radiolabeled with [α-^{32}P]dATP, using a commercial nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Labeled

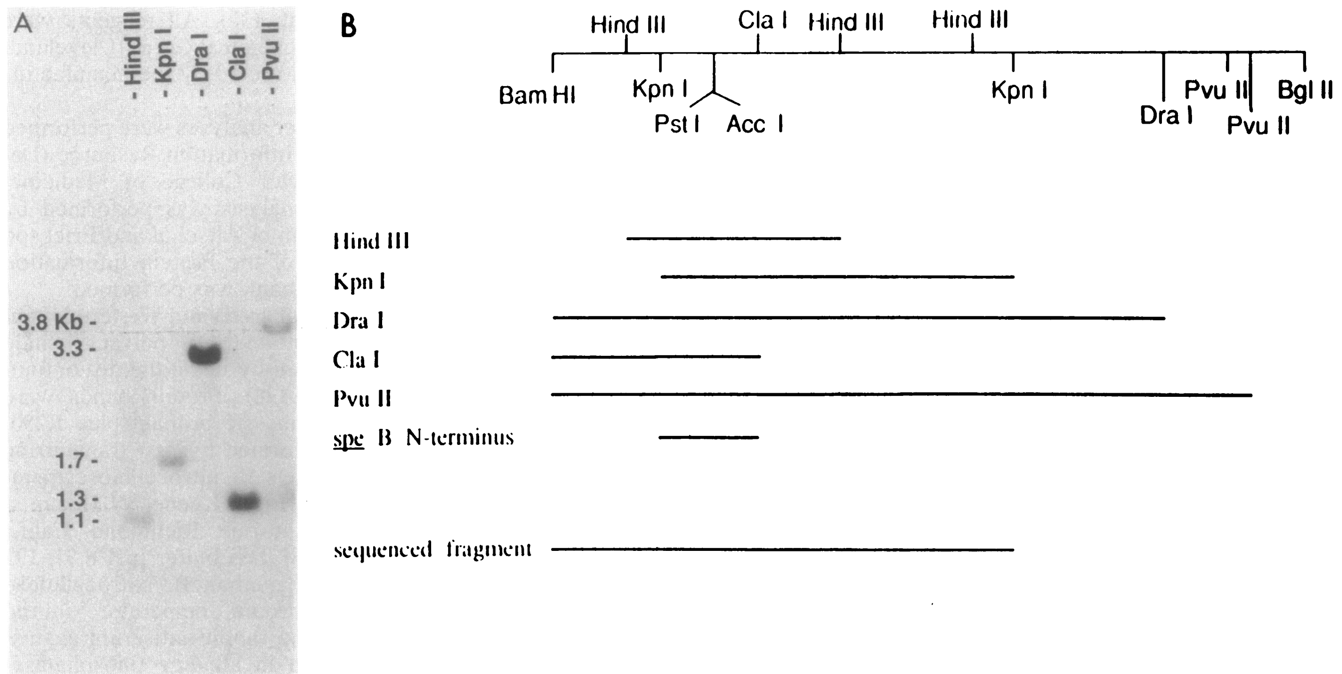

FIG. 1. (A) Autoradiograph of a restriction endonuclease-digested 4.5-kb *Bam*HI-*Bgl*II insert of pUMN713 hybridized with a radiolabeled *speB*-complementary oligonucleotide; (B) physical map of the insert showing the location of hybridized fragments. The insert was digested with *Hin*dIII, *Kpn*I, *Dra*I, *Cla*I, or *Pvu*II, as indicated at the top of the autoradiograph. In each case, the *speB*-complementary oligonucleotide probe hybridized to a single fragment, the size of which is indicated at the left. Overlap of the hybridized fragments indicates that the oligonucleotide hybridized within a 600-bp *Kpn*I-*Cla*I fragment. The indicated 2.4-kb *Bam*HI-*Kpn*I fragment was therefore sequenced.

probe DNA was separated from unincorporated [α-^{32}P]dATP by ethanol precipitation and denatured by incubation at 37°C for 5 min in 0.1 M sodium hydroxide.

Streptococcal chromosomal DNA used in hybridization experiments was obtained by treating cells with mutanolysin (Sigma) and SDS as described by Spanier and Cleary (38). Purified DNA was digested with *Eco*RI or *Pst*I, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose membranes (Schleicher & Schuell) (37). The nitrocellulose was incubated for 3 h at 65°C in prehybridization solution (5× SSC [SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% SDS, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.01% bovine serum albumin, 800 ng of denatured salmon sperm DNA). The membrane was then incubated for 16 h at 65°C in hybridization solution (5× SSC, 0.5% SDS, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.01% bovine serum albumin, 10% dextran sulfate) containing labeled probe. The nitrocellulose was washed in 2× SSC–0.5% SDS and dried. Bound probe was visualized by autoradiography.

RESULTS

Subcloning *speB*. The radiolabeled complementary oligonucleotide probe that had been constructed such that it would bind to the portion of *speB* which encodes the amino terminus of SPE B purified by the standard protocol hybridized to bands corresponding to the following subfragments of the 4.5-kb *Bam*HI-*Bgl*II insert: a 1.1-kb *Hin*dIII fragment, a 1.3-kb *Cla*I fragment, a 1.7-kb *Kpn*I fragment, a 3.3-kb *Dra*I fragment, and a 3.8-kb *Pvu*II fragment (Fig. 1). In each digestion, the probe hybridized to a single fragment. Knowledge of the restriction map of the 4.5-kb insert (8) allowed the localization of the hybridized fragments within this insert. Analysis of the overlap of the fragments determined that the hybridization portion of *speB* was contained within a 0.6-kb *Kpn*I-*Cla*I fragment (Fig. 1). A 2.4-kb *Bam*HI-*Kpn*I fragment consisting of the 0.6-kb *Kpn*I-*Cla*I subfragment as well as flanking DNA on both sides was isolated by agarose gel electrophoresis after restriction endonuclease digestion of pUMN713. The appropriate band was extracted from the gel and cloned into M13.

Nucleotide sequence of *speB*. The sequence of *speB* is shown in Fig. 2. The structural gene contained a 1,194-bp open reading frame beginning at an ATG start codon, ending with a TAG termination codon, and coding for a 398-residue protein. Ninety-five percent of the gene was sequenced in both directions, and all regions were sequenced at least twice. The open reading frame was preceded by putative −35 and −10 promoter regions and a typical Shine-Dalgarno sequence (AAGAGG). Palindromic sequences were located 3′ of the termination codon.

Primary structure of SPE B. Figure 2 shows the inferred amino acid sequence of SPE B based on nucleotide sequence data (molecular weight, 43,181). The peptide underscored by the solid line corresponds exactly to the amino-terminal sequence obtained from SPE B purified by the standard protocol. The peptide underscored by the dashed line corresponds to the amino-terminal sequence obtained from SPE B purified by the proteinase inhibitor purification procedure. Differential migration in SDS-polyacrylamide gels and subsequent Western immunoblots using protein prepared by both protocols was observed (Fig. 3). Anti-SPE B-reactive protein purified by the proteinase inhibitor purification protocol was heterogeneous, ranging in molecular weight from approximately 30,000 to 40,000. These data are consistent with SPE B being translated as a 43,181-dalton protein which, upon cleavage of the signal peptide, has a molecular

weight of 40,314. Proteolysis may occur, yielding a relatively stable 27,588-molecular-weight product.

Similarity studies. The inferred amino acid sequence of SPE B was compared with the published amino acid sequences of SPEs A and C, TSST-1, and the staphylococcal enterotoxins by Monte Carlo analysis. No significant similarity was found between SPE B and the other two SPEs or between SPE B and the staphylococcal enterotoxins, but possible similarity was found between SPE B and TSST-1. Examination using a homology program indicated that no significant similarity exists between small (10-amino-acid) regions of SPE B and the above-mentioned pyrogenic toxins.

Gerlach et al. have hypothesized that SPE B is identical to SPP (19). The inferred amino acid sequence of SPE B was therefore compared with the published amino acid sequence SPP as determined by Yonaha et al. (47) and Tai et al. (40) (Fig. 4). Of the 337 residues in SPP, 312 matched with residues in SPE B. Interestingly, the putative active site regions of SPP and SPE B differed in the location of a histidine residue.

Proteinase assay. SPE B was examined for its ability to proteolyze casein, an activity that has been demonstrated for SPP (18, 26). FITC-labeled casein was incubated with reduced SPE B purified from *E. coli* K-12 RR1(pUMN720), *S. pyogenes* 86-858, or *S. pyogenes* NY-5 by the standard protocol for as long as 6 h, but no proteolysis above that seen in the absence of SPE B was detected (data not shown). Substrate incubated with trypsin, however, exhibited fluorescence 2 to 17 times that of the negative control, depending on the length of the incubation. This assay has been reported to be 1,000 times more sensitive than the measurement of cleavage of casein peptides by A_{280} (42).

DNA hybridizations. A 612-bp internal DNA probe was constructed from *speB* and hybridized to *Eco*RI-digested chromosomal DNA purified from five group A streptococcal strains, 86-858, NY-5, C203S, C203U, and T19, commonly used in the laboratory. For each strain, the probe hybridized to DNA fragments of a single size (data not shown). To investigate whether this fragment contained more than one gene that could hybridize with the probe, chromosomal DNA from each strain was digested with *Pst*I, which digests once in the middle of *speB*. The probe hybridized to fragments of DNA of two different sizes, each fragment containing one half of *speB* (data not shown). For each strain no evidence was seen, which indicated that the probe was binding to more than one gene.

DISCUSSION

A total of 1,630 nucleotides consisting of *speB* and its 5'- and 3'-flanking regions was sequenced. A 1,194-bp open reading frame beginning with ATG was detected. Putative −35 and −10 promoter regions as well as a Shine-Dalgarno

```
  -35                              -10
GTTGTCAGTG TCAACTAACC GTGTTATTGT CTATTACCAT TCATGGTATC AGCGA

CATCG TATGATAACC ATACGATTCA GCTAAGTAAG GAGGTGTGTC CAATGTACCG

                                          S.D.
 TTAAAAGCAA ATGCAGTAGA TTAACTTATT TTGAAAGAGG TATAAAAAAA ATG
                                                        met

   4: AAT AAA AAG AAA TTA GGT ATC AGA TTA TTA AGT CTT TTA GCA TTA
      asn lys lys lys leu gly ile arg leu leu ser leu leu ala leu

  49: GGT GGA TTT GTT CTT GCT AAC CCA GTA TTT GCC*GAT CAA AAC TTT
      gly gly phe val leu ala asn pro val phe ala asp gln asn phe

  94: GCT CGT AAC GAA AAA GAA GCA AAA GAT AGC GCT ATC ACA TTT ATC
      ala arg asn glu lys glu ala lys asp ser ala ile thr phe ile

 139: CAA AAA TCA GCA GCT ATC AAA GCA GGT GCA CGA AGC GCA GAA GAT
      gln lys ser ala ala ile lys ala gly ala arg ser ala glu asp

 184: ATT AAG CTT GAC AAA GTT AAC TTA GGT GGA GAA CTT TCT GGC TCT
      ile lys leu asp lys val asn leu gly gly glu leu ser gly ser

 229: AAT ATG TAT GTT TAC AAT ATT TCT ACT GGA GGA TTT GTT ATC GTT
      asn met tyr val tyr asn ile ser thr gly gly phe val ile val

 274: TCA GGA GAT AAA CGT TCT CCA GAA ATT CTA GGA TAC TCT ACC AGC
      ser gly asp lys arg ser pro glu ile leu gly tyr ser thr ser

 319: GGA TCA TTT GAC GCT AAC GGT AAA GAA AAC ATT GCT TCC TTC ATG
      gly ser phe asp ala asn gly lys glu asn ile ala ser phe met

 364: GAA AGT TAT GTC GAA CAA ATC AAA GAA AAC AAA AAA TTA GAC ACT
      glu ser tyr val glu gln ile lys glu asn lys lys leu asp thr

 409: ACT TAT GCT GGT ACC GCT GAG ATT AAA CAA CCA GTT GTT AAA TCT
      thr tyr ala gly thr ala glu ile lys gln pro val val lys ser

 454: CTC CTT GAT TCA AAA GGC ATT CAT TAC AAC CAA GGT AAC CCT TAC
      leu leu asp ser lys gly ile his tyr asn gln gly asn pro tyr

 499: AAC CTA TTG ACA CCT GTT ATT GAA AAA GTA AAA CCA GGT GAA CAA
      asn leu leu thr pro val ile glu lys val lys pro gly glu gln

 544: TCT TTT GTA GGT CAA CAT GCA GCT ACA GGA TGT GTT GCT ACT GCA
      ser phe val gly gln his ala ala thr gly cys val ala thr ala

 589: ACT GCT CAA ATT ATG AAA TAT CAT AAT TAC CCT AAC AAA GGG TTG
      thr ala gln ile met lys tyr his asn tyr pro asn lys gly leu

 634: AAA GAC TAC ACT TAC ACA CTA AGC TCA AAT AAC CCA TAT TTC AAC
      lys asp tyr thr tyr thr leu ser ser asn asn pro tyr phe asn

 679: CAT CCT AAG AAC TTG TTT GCA GCT ATC TCT ACT AGA CAA TAC AAC
      his pro lys asn leu phe ala ala ile ser thr arg gln tyr asn

 724: TGG AAC AAC ATC CTA CCT ACT TAT AGC GGA AGA GAA TCT AAC GTT
      trp asn asn ile leu pro thr tyr ser gly arg glu ser asn val

 769: CAA AAA ATG GCG ATT TCA GAA TTG ATG GCT GAT GTT GGT ATT TCA
      gln lys met ala ile ser glu leu met ala asp val gly ile ser

 814: GTA GAC ATG GAT TAT GGT CCA TCT AGT GGT TCT GCA GGT AGC TCT
      val asp met asp tyr gly pro ser ser gly ser ala gly ser ser

 859: CGT GTT CAA AGA GCC TTG AAA GAA AAC TTT GGC TAC AAC CAA TCT
      arg val gln arg ala leu lys glu asn phe gly tyr asn gln ser

 904: GTT CAC CAA ATT AAC CGT AGC GAC TTT AGC AAA CAA GAT TGG GAA
      val his gln ile asn arg ser asp phe ser lys gln asp trp glu

 949: GCA CAA ATT GAC AAA GAA TTA TCT CAA AAC CAA CCA GTA TAC TAC
      ala gln ile asp lys glu leu ser gln asn gln pro val tyr tyr

 994: CAA GGT GTC GGT AAA GTA GGC GGA CAT GCC TTT GTT ATC GAT GGT
      gln gly val gly lys val gly gly his ala phe val ile asp gly

1039: GCT GAC GGA CGT AAC TTC TAC CAT GTT AAC TGG GGT TGG GGT GGA
      ala asp gly arg asn phe tyr his val asn trp gly trp gly gly

1084: GTC TCT GAC GGC TTC TTC CGT CTT GAC GCA CTA AAC CCT TCA GCT
      val ser asp gly phe phe arg leu asp ala leu asn pro ser ala

1129: CTT GGT ACT GGT GGC GGC GCA GGC GGC TTC AAC GGT TAC CAA AGT
      leu gly thr gly gly gly ala gly gly phe asn gly tyr gln ser

1174: GCT GTT GTA GGC ATC AAA CCT TAG TAT GGAAATGCAT TTCGTTAGAA CA
      ala val val gly ile lys pro ***

      GAACCTGA GGCACGCGAT AGCTGAAACC TTTTTGTGCC GAAAACACAC AAAGCAA

      AAG CCCCTATCGT GTGCAGCAGT TAAGCTATCC ATCAGACACA GATCACTCAG G

      TGGTGAGCT ATACATCTAT GCTTTGTCTC CTGCTGGATT TATCATCGTA TCAGGA

      GACA CCAGAGCGCA CACCATTTTA GGCTATTCTT TTGATAATAA CCTGGACCTC

      AACCATGATA ATGTCAGAAG TATGGTAG
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FIG. 2. Nucleotide and deduced amino acid sequences of *speB* and SPE B, respectively (GenBank accession no. M35110). Numbering is in reference to the ATG start codon. Possible promoter (−10 and −35) and Shine-Dalgarno (S.D.) sequences are indicated. The probable cleavage site between the signal peptide and the mature protein is marked by the asterisk following residue 27. The amino-terminal sequence of SPE B purified by the proteinase inhibitor protocol is identical to the sequence indicated by the dashed underline, and the amino-terminal sequence of SPE B purified by the standard protocol is identical to the sequence indicated by the solid underline. Overlined nucleotides indicate palindromic sequences located 3' of the translation stop codon.

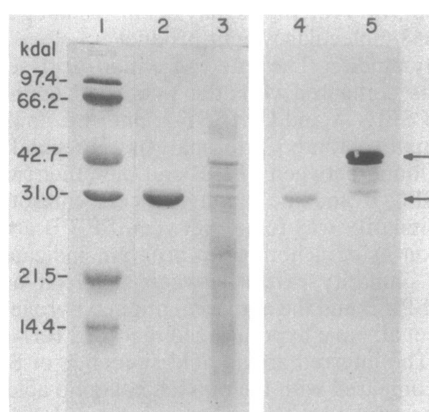

FIG. 3. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of SPE B purified by the standard protocol (lanes 2 and 4) and the proteinase inhibitor protocol (lane 3 and 5). Lane 1 contains molecular weight standards of the sizes indicated (in kilodaltons [kdal]) at the left. Lanes 1 to 3 contain proteins that were electrophoresed and stained with Coomassie blue. Lanes 4 and 5 contain proteins that were electrophoresed, transferred to nitrocellulose, and reacted with SPE B antiserum as described in Materials and Methods. The arrows indicate bands that may correspond to the 40,314-molecular-weight protein and the 27,588-molecular-weight breakdown product.

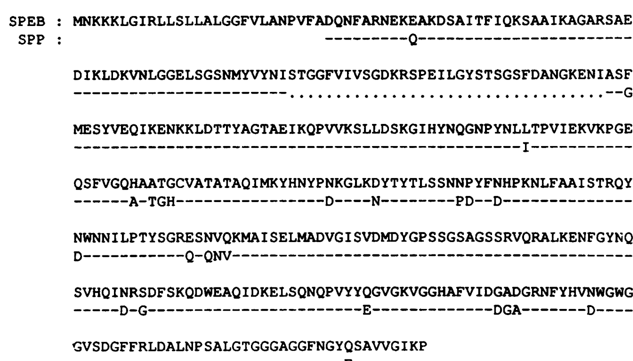

FIG. 4. Amino acid sequence similarity between SPE B and SPP. The sequence of SPE B is that inferred from the nucleotide sequence of *speB*. The sequence of SPP is that reported by Tai et al. (40) and Yonaha et al. (47) using protein sequencing techniques. Matched amino acids (—) and gaps (...) are indicated.

sequence were found 5′ of this start codon, indicating that it is probably the translational start site of *speB*. As is the case for some of the other PTs, a palindromic sequence which may be involved in transcription termination was found 3′ of the stop codon of *speB* (30).

Protein studies indicate that SPE B is initially translated as a 398-residue protein. Cleavage of a 27-amino-acid signal peptide yields a mature 371-residue protein with a molecular weight of 40,314. Subsequent proteolysis results in the formation of multiple smaller intermediates and eventually a relatively stable 253-residue breakdown product with a molecular weight of 27,588. Earlier reports indicating that the size of SPE B was approximately 30,000 daltons (8, 20) may have resulted from isolation of this breakdown product rather than the intact protein.

Despite shared biological activities, SPE B is not significantly similar to the other PTs with the possible exception of TSST-1, the toxin which is considered the most distantly related of all the previously sequenced PTs. Monte Carlo analysis of the mature amino acid sequences of the PTs indicates that SEB, SEC1, SEC2, and SPE A form a closely related cluster of toxins, that SEA, SED, and SEE form another cluster, and that TSST-1 and SPE B are much less closely related to the other PTs (21).

The comparison of the amino acid sequence of SPE B with the published sequence of SPP indicates that the proteins are closely related. We believe that many of the differences are due to protein sequencing difficulties in the earlier studies. The SPE B and SPP sequences differ at 13 locations by a single residue. Ten of these differences are glutamine-glutamate or asparagine-aspartate interchanges. It is difficult to distinguish these pairs by protein sequencing. The most significant difference between the two sequences is the omission of 34 residues (serine 84 through isoleucine 117) in the previously published SPP sequence. Examination of the technique used to sequence this portion of SPP (42) indicates that this region was contained in only one of the multiple overlapping peptides used to sequence SPP and that this portion of the peptide was sequenced near the resolution limit of the automated Edman degradation technique used. Furthermore, the omitted region is flanked by asparagine-isoleucine pairs, contributing to possible incorrect overlapping of sequenced peptides. The reported sequence of SPP dictates that it has a molecular weight of 37,000. The addition of the omitted region, assuming that it is an artifact of protein sequencing, would yield a protein with a molecular weight of 40,314. The estimate of the molecular weight of SPP by ultracentrifugation (26), amino acid composition studies (26), and SDS-polyacrylamide electrophoresis (19) as greater than 40,000 supports the conclusion that the actual protein sequence of SPP is larger than that reported. Several of the other sequence differences between SPE B and SPP are likely to reflect actual differences at the nucleotide level. For example, in four different regions, including the active-site area, the inferred amino acid sequence of SPE B was identical to that of SPP except that the amino acids were in a different order.

We believe that the evidence indicates that SPE B and SPP are variants of the same protein. The two proteins have similar isoelectric focusing patterns (2, 25), share epitopes (19), and have amino acid sequences whose major differences can be explained by protein sequencing artifacts. The remaining minor differences may indicate that SPE B and SPP exist as multiple subtypes. A precedent for this among the PTs is already known in SEC (subtypes SEC1, -2, and -3) (39). The lack of proteinase activity associated with SPE B purified from strain 86-858 may be a consequence of the placement of a histidine molecule five residues toward the amino terminus of SPE B, from where it has been reported to occur in the active site of SPP (27, 40). We believe that this change could account for the lack of proteolytic activity in SPE B purified from strain 86-858 but does not justify designating SPE B as a protein different from SPP.

The DNA hybridization results further indicate that SPE B and SPP are the same protein. If these proteins were the same, it would be expected that one gene would give rise to the protein, whereas if the proteins were distinct, one would expect that they would be encoded by two different genes and that both genes could exist within the same organism. Furthermore, the amino acid sequence similarities between the two proteins indicate that the 612-bp probe used in hybridization experiments should hybridize to genes for both proteins. In this study, we observed that the probe hybridized to a single gene in each of five strains tested even though

one of the strains, T19, has been reported to produce both SPP and SPE B (19).

The evidence presented above indicates that two subtypes of SPE B exist, one with and one without proteolytic activity. Both exhibit properties characteristic of PTs: pyrogenicity, skin rash production, and lymphocyte mitogenicity (2, 14, 19). In addition, one subtype, designated SPP, also has proteolytic activity, whereas the other subtype, which includes SPE B from *S. pyogenes* 86-858 and NY-5, does not. Additional studies will be necessary to investigate the effects of this proteolytic activity on the function of SPE B as a virulence factor.

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