INFECTION AND IMMUNITY, Apr. 1986, p. 144–150
0019-9567/86/040144-07$02.00/0

Vol. 52, No. 1

Nucleotide Sequence of the Type A Streptococcal Exotoxin (Erythrogenic Toxin) Gene from *Streptococcus pyogenes* Bacteriophage T12

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The gene specifying type A streptococcal exotoxin (*speA*), also known as erythrogenic toxin, was cloned from the *Streptococcus pyogenes* bacteriophage T12 genome and analyzed by nucleotide sequencing. The *speA* gene consists of 753 base pairs and codes for a 29,244-molecular-weight protein. The *speA* gene product contains a putative 30-amino acid signal peptide, resulting in a molecular weight of 25,787 for the secreted protein. A possible promoter and ribosome-binding site are present in the region upstream from the *speA* gene, and a transcriptional terminator is located 69 bases downstream from the translational termination codon. The amino acid sequence of the carboxy-terminal portion of the type A streptococcal exotoxin exhibits extensive homology with the carboxy terminus of *Staphylococcus aureus* enterotoxins B and C1.

Type A streptococcal exotoxin (*speA*), also known as erythrogenic toxin, is a phage conversion product secreted by bacteriophage-infected *Streptococcus pyogenes*. A number of biological activities such as skin reactivity, pyrogenicity, enhancement of host susceptibility to lethal endotoxin shock, lymphocyte mitogenicity, and alteration of immunoglobulin production have been attributed to the *speA* gene product (for reviews, see references 1 and 48).

Attempts to physically characterize the type A streptococcal exotoxin and study its effects on the host and the course of streptococcal disease have been hampered by the difficulty in isolating the protein in a pure and undegraded form. Nauciel et al. (35) first purified the type A streptococcal exotoxin by column chromatography and performed amino acid determinations on the 30,500-molecular-weight toxin. Further purification schemes utilized by Kim and Watson (17) and Gerlach et al. (4) resulted in purification of type A exotoxin with molecular weights of 29,400 and 28,000, respectively. Cunningham et al. (3) purified type A exotoxin from *S. pyogenes* NY5-10 culture supernatants by differential solubility in ethanol and acetate-buffered saline followed by ion-exchange chromatography. Two distinct fractions isolated by this method had molecular weights of 8,000 and 5,500 and were found to be pyrogenic and to alter the antibody response. Houston and Ferretti (11) isolated an 8,500-molecular-weight type A streptococcal exotoxin which induced the erythematous skin reaction. Discrepancies observed in the molecular weight and the amino acid composition of the purified type A streptococcal exotoxin are indicative of the difficulties encountered in isolating pure type A exotoxin. To circumvent these problems, recombinant DNA techniques were employed to isolate the gene for type A streptococcal exotoxin and to introduce it into *Escherichia coli* (13, 49). In the present communication, we report the nucleotide sequence of the *speA* gene and the deduced amino acid composition of the type A streptococcal exotoxin.

MATERIALS AND METHODS

Bacteria, bacteriophage, and phage vectors. A 1.8-kilobase (kb) phage T12 DNA fragment which contains the gene for type A streptococcal exotoxin was ligated to pBR322 and used to transform *E. coli* HB101 as described previously (49). The resulting plasmid, p1179, was the source of the type A streptococcal exotoxin (*speA*) gene. *E. coli* JM101 and JM109 served as recipients for transfection experiments with the *E. coli* phage M13 vectors (29, 51). *E. coli* phage vectors M13mp8, M13mp9, M13mp10, M13mp18, and M13mp19 were utilized for subcloning and sequencing experiments (30, 37). M13mp8 and M13mp9 were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.); *Sma*I-digested alkaline phosphatase-treated M13mp10 was obtained from Amersham Corp. (Arlington Heights, Ill.); and M13mp18 and M13mp19 were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Media. *E. coli* HB101 containing the chimeric plasmid p1179 was grown in LB medium containing 50 μg of ampicillin per ml. *E. coli* JM101 and JM109 were grown in 2× YT broth (33). Soft agar overlays consisted of 2× YT broth supplemented with final concentrations of 0.75% agar, 0.33 mM isopropyl-β-D-thiogalactopyranoside, and 0.02% 5-bromo-4-chloro-3-indolyl-β-galactoside when differentiating between recombinant and nonrecombinant phage.

Enzymes and chemicals. Restriction enzymes were purchased from Bethesda Research Laboratories and were used according to the specifications of the manufacturer. T4 DNA ligase was purchased from Amersham or Bethesda Research Laboratories. The Klenow fragment of DNA polymerase and the M13 15-base primer were purchased from Bethesda Research Laboratories. The deoxy- and dideoxynucleotide triphosphates were purchased from P-L Biochemicals and the [α-^{32}P]dATP was purchased from New England Nuclear Corp. (Boston, Mass.) or Amersham. Isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-galactoside were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Subcloning of *speA* into M13 vectors for nucleotide sequencing. The *speA* gene was obtained for subcloning experiments by digestion of p1179 with *Sal*I and *Eco*RI followed by electrophoresis and isolation of the 1.8-kb fragment from 0.8% type VII agarose gels as described previously (19). The 1.8-kb DNA fragment was ligated to *Sal*I-*Eco*RI-digested

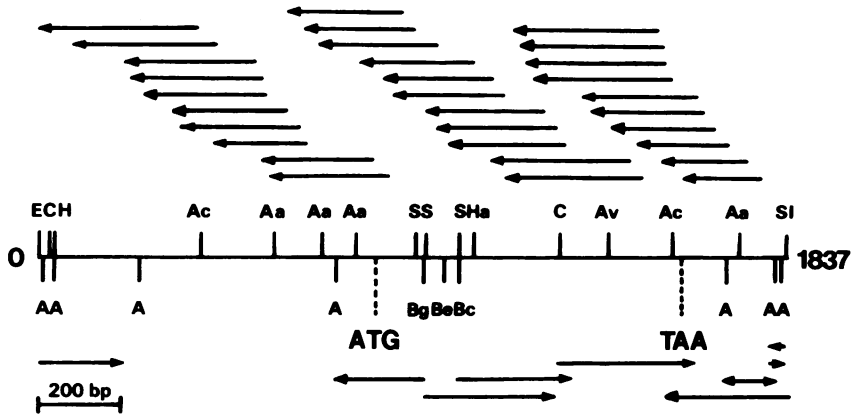

FIG. 1. Partial restriction map and sequencing strategy for the type A streptococcal exotoxin gene, *speA*, which is contained in a 1,837-base-pair (bp) fragment isolated from p1179 (49). The arrows indicate the direction and extent of sequence derived from each independent clone. The arrows above the map represent *Bal* 31-generated clones; arrows below the map represent clones obtained from restriction enzyme digestion of the isolated fragment. The restriction sites are: A, *Alu*I; Aa, *Aha*III; Ac, *Acc*I; Av, *Ava*II; Bc, *Bcl*I; Be, *Bst*EII; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; P, *Pst*I; S, *Sau*3A; and SI, *Sal*I. The positions of the initiation (ATG) and termination (TAA) codons of *speA* are shown.

M13mp8 and M13mp9 and transfected into *E. coli* JM101 by a modification of the procedure of Mandel and Higa (26) as described in the Amersham cloning and sequencing handbook. Colorless plaques were used to infect *E. coli* JM101. The resulting chimeric replicative form of the phage was isolated by the alkaline lysis method and analyzed to confirm the presence of the 1.8-kb DNA fragment (2). Also, the 1.8-kb isolated fragment was digested with *Alu*I, ligated to *Sma*I-digested, alkaline phosphatase-treated M13mp10, and transfected into *E. coli* JM101.

Subsequent subcloning experiments utilized the replicative form of the M13mp8 or M13mp9 phage containing the 1.8-kb insert as a source of 1.8-kb fragment. To obtain a large amount of the 1.8-kb fragment for further cloning experiments, and to unidirectionally degrade this fragment with *Bal* 31, a modification of the technique of Gilmore et al. (5) was employed. Briefly, 5 mg of the replicative form of M13mp8 containing the 1.8-kb fragment was digested with *Pst*I (adjacent to the *Sal*I site in the M13 polylinker) and *Eco*RI, and the 1.8-kb fragment was isolated by centrifugation through a 10 to 40% sucrose gradient (27). The 1.8-kb fragment was self-ligated overnight in the presence of T4 DNA ligase to produce long polymers. These polymers were subsequently digested with *Pst*I to produce dimers of the 1.8-kb fragment linked at the *Eco*RI digest site. The dimers were digested with *Bal* 31, and at 5-min intervals, samples were removed and the *Bal* 31-digested DNA was precipitated with ethanol. The DNA was further digested with *Eco*RI to produce fragments containing *Eco*RI and *Bal* 31 ends. Finally, this processed DNA was ligated to *Sma*I-*Eco*RI-digested M13mp18 and transfected into *E. coli* JM109.

Additional clones were prepared for sequencing by digestion of the isolated fragment with *Sau*3A, *Cla*I, or double digestion with *Bgl*II and *Alu*I and subsequent ligation to *Pst*I-*Bam*HI-digested M13mp19, *Acc*I-*Pst*I-digested M13 mp9, and *Sma*I-*Bam*HI-digested M13mp18, respectively.

Colorless plaques obtained in each cloning experiment were reinfected into *E. coli*, and the cells were screened for the replicative form by the alkaline lysis method to confirm the size and presence of the insert before preparation of single-stranded DNA for use in sequencing.

Nucleotide sequencing. Recombinant phage were used to infect *E. coli* JM101 or JM109 for preparation of recombinant phage stocks or template DNA as described previously (44).

Sequencing reactions were performed by the Sanger dideoxy chain termination method (45) according to the procedures described by Amersham. All sequences were confirmed from at least two overlapping clones, and 94% of the *speA* gene sequence was determined on both strands. The sequence information was analyzed by the James M. Pustell DNA/protein sequencing program obtained from International Biotechnologies, Inc.

Immunological assay for presence of type A streptococcal exotoxin. *E. coli* JM101 and JM109 containing recombinant phage were tested for type A streptococcal exotoxin production as described previously (49) with minor modifications. Single-stranded recombinant phage containing the 1.8-kb fragment or *Bal* 31-degraded derivatives were used to infect *E. coli* JM101 or JM109, respectively, in 2× YT broth. After overnight growth, cells were pelleted by centrifugation, and cell sonic extracts were tested for reactivity with specific type A streptococcal exotoxin antibody as described previously (49).

RESULTS AND DISCUSSION

The 1.8-kb fragment containing the type A streptococcal exotoxin gene obtained from bacteriophage T12 (49) was isolated, digested with various restriction enzymes, and ligated to the appropriate M13 phage vectors to obtain template DNA for sequencing reactions. The staggered array of fragments generated by unidirectional digestion with *Bal* 31 ensured the correct alignment of the sequences and provided sequence duplication from both strands for each region (see Fig. 1).

A restriction enzyme digestion map of the 1.8-kb fragment containing the gene for type A streptococcal pyrogenic exotoxin (*speA*) is shown in Fig. 1. The *speA* gene contains single restriction sites for the enzymes *Cla*I, *Ava*II, *Bcl*I, *Bgl*II, *Bst*EII, and *Hae*III.

Examination of the nucleotide sequence revealed that the *speA* gene is encoded by 753 base pairs located between nucleotides 814 and 1567 on the cloned fragment (Fig. 2). A typical ribosome-binding site (RBS) sequence GGAGG, sim-

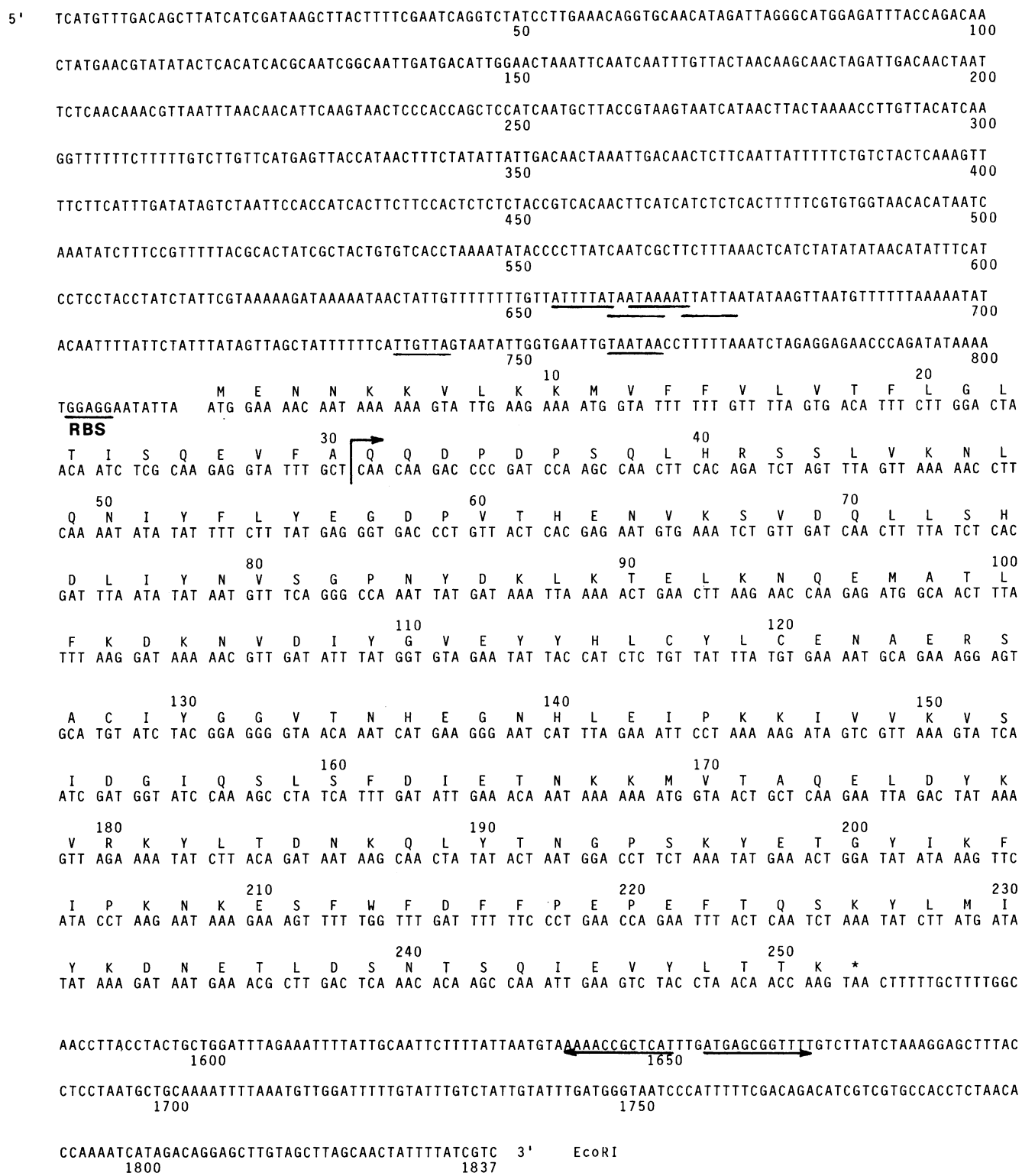

FIG. 2. Nucleotide sequence of *speA* and flanking regions. Numbering begins at the *Eco*RI site at the 5′ end of the sequence. The deduced amino acid sequence of the type A streptococcal exotoxin is given above the nucleotide sequence. A possible cleavage site for the removal of the signal peptide is designated by a bent arrow. Putative promoter and RBS sequences are underlined. Palindromic sequences upstream from the promoter are underlined as is a possible transcription termination sequence in the downstream region of *speA*.

ilar to *Bacillus* (7, 9, 18, 32, 36, 38, 40, 41, 50), *Staphylococcus* (7, 28, 43), *Streptococcus* (21, 25), and *E. coli* (6) RBS sequences, is present 7 bases upstream from the ATG translation initiation codon. A putative −10 region TAATAAC sequence similar to the *Bacillus subtilis* phage *spo1-15* early gene promoter (22) and the *Streptococcus equisimilis* streptokinase gene promoter (25) is located 35 base pairs upstream from the RBS sequence. A probable −35 region TTGTTA sequence is located 17 bases upstream from the proposed −10 promoter region. Comparison of this −35 region of the *speA* gene with other promoters of gram-positive and gram-negative organisms reveals that the *speA* −35 regions is identical to the promoter of the *Bacillus amyloliquefaciens* α-amylase gene, TTGTTA (39). In general, the sequences of the −10 and −35 regions of the *speA* gene conform to the consensus sequences TTGACA and TATAAT of promoters recognized by *E. coli* RNA polymerase (9, 42). The strongly conserved TTG of the *E. coli* −35 region and the invariant T of the *E. coli* −10 region are present in these regions of the *speA* gene. The 17-base spacer region between the −35 and −10 regions of *speA* follows the pattern reported for numerous *Bacillus* phage and chromosomal genes (7, 22, 23, 32, 34, 38, 41, 50), *Staphylococcus* genes (24, 43), and *E. coli* genes (10, 42). In addition, the seven bases between the RBS sequence and the translation initiation codon is the preferred distance in *E. coli* genes (10, 42) and in most genes of gram-positive organisms (8, 28, 32, 43, 50). Transcription in *E. coli* JM101 is probably initiated from these promoters rather than from the M13 phage promoters since the toxin gene is expressed equally in both orientations and induction by isopropyl-β-D-thiogalactopyranoside is not necessary for toxin gene expression. The similarity of the presumed *speA* promoter region to the promoter region recognized by *E. coli* RNA polymerase probably accounts for the comparable expression of the *speA* gene in *E. coli* and *S. pyogenes*.

The region 100 bases upstream from the −35 promoter region, spanning nucleotides 640 to 740, is unusually AT rich and has an A+T content of 89% compared with an A+T content of 70% for the coding region of the *speA* gene. This region also contains several potential inverted repeats. Similar regions have been reported in nucleotide sequences of staphylokinase (43), streptokinase (25), *Bacillus licheniformis* penicillinase (36), diphtheria toxin (14), and *B. subtilis* phage *spo-15*, *spo-26*, and *spoVG* genes (32). It has been suggested by several investigators that these regions play a role in gene regulation, promoter recognition, or efficient promoter utilization. Moran et al. (31) have shown by deletion analysis that the AT-rich region upstream from the −35 region of the *spoVG* gene is important for its use by sigma-37 RNA polymerase.

Translation of the type A exotoxin gene terminates at nucleotide 1567. An extended inverted repeat capable of stem-loop formation is located 69 bases downstream from the TAA stop translation codon. This stem-loop structure consists of a perfectly matched 12-base stem separated by a 3-base loop. The GC-rich stem followed by an AT-rich region probably serves as a transcription terminator (42).

The deduced *speA* gene product is composed of 251 amino acids and has a molecular weight of 29,244 as calculated from the nucleotide sequence. As expected for a secreted protein, a probable signal peptide of 30 amino acids can be identified from the amino acid sequence. Removal of the leader peptide by cleavage between glycine and alanine such as occurs in other secreted proteins (39) would result in a type A exotoxin with a molecular weight of 25,787. The proposed leader peptide is initiated by a string of 10 hydrophilic amino acids followed by a region of 19 hydrophobic amino acids. A hydropathy plot computed from the sliding average of the hydropathy values of nine amino acids (20) supports the presence of a signal peptide (data not shown). Several extracellular proteins of gram-positive organisms such as *B. amyloliquefaciens* α-amylase (40), *B. licheniformis* penicillinase (18), *B. amyloliquefaciens* subtilisin (50), and *Staphylococcus* protein A (24) have relatively longer signal peptides than exported proteins of gram-negative organisms. The 30-amino acid signal peptide proposed for the *speA* gene product is in the same general size range as those reported for other gram-positive organisms (39).

The deduced amino acid composition of the *speA* gene product in the presence and absence of the leader peptide is presented in Table 1. The mature type A exotoxin contains three cysteine residues and is rich in lysine, leucine, and the dicarboxylic amino acids, glutamic acid and aspartic acid. The amino acid composition derived from the nucleotide sequence closely parallels data reported by Gerlach et al. (4), with a few exceptions. The nucleotide sequence indicates that the mature type A exotoxin contains 1.4% cysteine, 0.5% tryptophan, and 4.2% glycine compared with 0.2% cysteine, 1.45% tryptophan, and 5.4% glycine obtained from protein sequencing. These discrepancies may be due to the different sources of type A exotoxin. Bacteriophage T12 induced from *S. pyogenes* T253 served as the source for the *speA* gene used for DNA sequence determination, while amino acid data reported by Gerlach et al. (4) were based on *speA* gene product isolated from *S. pyogenes* NY5-10.

E. coli cells infected with single-stranded M13 phage containing the *speA* gene synthesized type A streptococcal exotoxin intracellularly. This exotoxin could be identified by immunological reaction with specific antibody to the type A

TABLE 1. Amino acid composition of type A streptococcal exotoxin[a]

Amino acid	From DNA sequence (%)		From protein analysis (%)[b]
	With signal peptide	Without signal peptide	
Ala	2.0	1.9	2.8
Val	7.6	6.4	5.9
Leu	9.6	9.1	8.6
Ile	5.6	5.9	5.1
Pro	3.6	4.2	4.0
Met	2.0	1.4	1.5
Phe	5.2	4.1	3.8
Trp	0.4	0.5	1.45
Gly	4.0	4.2	5.4
Ser	6.4	6.9	7.1
Thr	6.4	6.5	6.7
Cys	1.2	1.4	0.2
Tyr	6.8	7.7	6.8
Asn	7.2	7.2	13.5[c]
Gln	4.8	5.0	12.3[c]
Asp	6.0	6.8	—[c]
Glu	7.6	7.7	—[c]
Lys	10.4	10.0	10.2
His	2.4	2.8	2.7
Arg	1.2	1.4	1.7

[a] Values for individual amino acids are presented as a percentage of the total number of amino acids.

[b] Protein analysis data were reported by Gerlach et al. (4).

[c] Values for Asp and Glu are included in the values calculated for Asn and Gln, respectively, in the protein analysis.

FIG. 3. Comparison of the amino acid sequences of type A streptococcal exotoxin (speA), staphylococcal enterotoxin B (SEB), and staphylococcal enterotoxin C1, (SEC1). Boxes are used to indicate identical residues. Underlined residues are common in one of the two staphylococcal enterotoxins. The sequence of enterotoxin B is taken from Huang and Bergdoll (12), and the sequence of enterotoxin C1 is taken from Schmidt and Spero (46).

exotoxin. Testing of *E. coli* cell sonic extracts derived from infection with various *Bal* 31-digested M13 *speA* phage clones for toxin production allowed localization of the boundary of the toxin gene and identification of the major antibody-reactive site. Digestion of the 1.8-kb fragment by *Bal* 31 sequentially removed nucleotides downstream from the coding region of the gene. Removal of bases to nucleotide 1540 resulted in the inability to detect toxin immunologically. These results indicate that the major immunogenic determinant of type A streptococcal exotoxin is located at the carboxy-terminal portion of the toxin protein. Alternatively, loss of the hydrophilic carboxy-terminal portion of the type A exotoxin may cause improper folding of the protein leading to conformational distortions and sequestering of the antigenic determinants responsible for reactivity with specific antibody.

A comparison of the amino acid sequence of the *speA* gene product with other known sequenced proteins revealed an interesting partial homology with staphylococcal enterotoxins B (12) and C1 (46), especially striking at the carboxy-terminal portions of these proteins (Fig. 3). *Staphylococcus aureus* enterotoxins have been reported to cause pyrogenicity and lymphocyte mitogenicity and to enhance susceptibility to endotoxin shock, in addition to their emetic activity (15, 46). These properties are similar to those attributed to the type A streptococcal exotoxin. The mitogenic activity of *S. aureus* enterotoxin C1 has been assigned to the amino-terminal portion, while two of the major antigenic determinants have been assigned to the carboxy-terminal portion of the protein (47). Further studies utilizing site-specific mutagenesis must be undertaken to determine whether the *S. aureus* enterotoxins and the type A streptococcal exotoxin share common active sites. Although the gene for enterotoxin B has been located on the *S. aureus* chromosomal genome, it has been suggested that the enterotoxin B gene is associated with a hitchhiking transposon (16). Nucleotide sequence data of the *S. aureus* enterotoxin B gene may dismiss or support possible evolutionary relatedness between the type A streptococcal exotoxin and *S. aureus* enterotoxin B.

The nucleotide sequence of the *speA* gene and the deduced amino acid determination provide the basis for further studies on the molecular role of the type A exotoxin in streptococcal disease. Site-specific mutagenesis of the cloned *speA* gene fragment will allow determination of the immunogenic determinants and regions of the protein responsible for skin reactivity, pyrogenicity, mitogenicity, and macrophage cytotoxicity.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 9304 from the National Institutes of Health.

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