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Molecular Characterization and Phylogenetic Distribution of the Streptococcal Superantigen Gene (*ssa*) from *Streptococcus pyogenes*

KRISTIN B. REDA,[1] VIVEK KAPUR,[2] JOSEPH A. MOLLICK,[1] JAMES G. LAMPHEAR,[1] JAMES M. MUSSER,[1,2] AND ROBERT R. RICH[1,3]*

Departments of Microbiology and Immunology,[1] Pathology,[2] and Medicine,[3] Baylor College of Medicine, Houston, Texas 77030

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A striking increase in the frequency and severity of invasive infections caused by *Streptococcus pyogenes* has occurred in recent years. Among these diseases is streptococcal toxic-shock-like syndrome (TSLS), a condition characterized by fulminant soft-tissue destruction and multiorgan failure. Streptococcal superantigen (SSA), a superantigen isolated from a TSLS-inducing, serotype M3 *S. pyogenes* strain, has recently been identified. We here describe the cloning, sequencing, and phylogenetic distribution of the SSA structural gene. The 783-bp open reading frame encodes a predicted 260-amino-acid protein that is similar in size to several other bacterial superantigens. The deduced sequence of the mature protein is 60.2% identical to that of staphylococcal enterotoxin B but only 49% identical to that of streptococcal pyrogenic exotoxin A. Southern blot and PCR analysis of 138 group A streptococcal strains representing 65 M protein serotypes and 15 nontypeable isolates identified *ssa* in 68 strains from 10 distinct clonal lineages. All *ssa*-positive clones expressed SSA. Of the two clones associated with TSLS, the ET 2-M3 lineage, but not the ET 1-M1 lineage, carried the SSA gene. Further analysis of the ET 2-M3 lineage found evidence for temporal variation in *ssa* association. Contemporary ET 2-M3 disease isolates had *ssa*, but two older isolates of this clone recovered in 1910 and 1920 lacked the gene. The clonal and temporal distribution patterns of *ssa* suggest a relatively recent acquisition of this superantigen-encoding gene by the ET 2-M3 lineage, perhaps by horizontal transfer and recombination.

The prevalence of severe *Streptococcus pyogenes* infections such as rheumatic fever and toxic-shock-like syndrome (TSLS) increased in the mid-1980s (6, 24, 50, 55). Epidemiologic studies have found this disease resurgence to be associated with changes in serotype frequency distribution and pyrogenic exotoxin production (22, 25, 30, 40, 47, 53). For example, in the United States, serotypes M1, M3, and M18 each accounted for a significantly greater proportion of isolates typed by the Centers for Disease Control in 1980 to 1988 than in 1972 to 1979 (47). These serotypes were also expressed by 71% of rheumatic fever isolates recovered in 1986 to 1988 (30). Strains expressing M1 or M3 serotype and streptococcal pyrogenic exotoxin A (SPE A) caused nearly two-thirds of contemporary TSLS episodes (40), and SPE A expression correlated with the clinical signs of shock and organ involvement (53). However, the lack of complete association between severe streptococcal disease and M protein serotype or SPE A production suggests that additional bacterial products are involved in pathogenesis (13).

SPE A and the related toxin SPE C belong to a family of bacterial protein superantigens that are expressed primarily by *Staphylococcus aureus* and *S. pyogenes* (14, 35). This toxin family also includes staphylococcal enterotoxins A through F (SEA through SEF) (2, 5, 8, 9, 12, 26, 29, 45) and toxic shock syndrome toxin 1 (TSST-1) (7). Although made by two different bacterial genera, the toxins have significant amino acid homology and share several biological functions, including pyrogenicity, enhancement of susceptibility to endotoxin shock, and suppression of immunoglobulin production (3). As superantigens, these molecules bind to major histocompatibility complex (MHC) class II molecules (37) to form a complex that interacts with T-cell subsets bearing specific Vβ regions on their T-cell receptors (31). Superantigens stimulate MHC class II-dependent T-cell proliferation and release of lymphokines such as interleukin 1, interleukin 2, tumor necrosis factor alpha, and gamma interferon (10, 21, 36). These immunomodulatory effects may cause some of the signs and symptoms observed in severe staphylococcal and streptococcal infections.

The possibility that an *S. pyogenes* TSLS isolate expressed an uncharacterized superantigen was recently explored (39) and resulted in the purification of an ~28-kDa protein, designated streptococcal superantigen (SSA), from culture supernatants. SSA stimulated proliferation of human T cells bearing Vβ1, Vβ3, Vβ5.2, and Vβ15 in an MHC class II-dependent manner. N-terminal sequencing found the first 24 residues of SSA to be 62.5% identical to staphylococcal superantigens SEB, SEC1, and SEC3 (39). Immunoblot analysis of culture supernatants detected SSA production by only 3 of 15 group A streptococcal clonal lineages. Among these phylogenetic lineages was the ET 2-M3 clone, one of the two clones responsible for the majority of recent cases of severe streptococcal disease (40).

In order to define the phylogenetic distribution of SSA and to characterize its structural relationship to other superantigens, we have cloned the SSA gene from a TSLS isolate. Nucleotide sequence comparisons show that *ssa* has considerable homology with *seb*, *sec1*, *sec2*, and *sec3*. *ssa* was found in 10 streptococcal clonal lineages that in several cases are highly divergent in chromosomal character, as indexed by multilocus enzyme electrophoresis (48). The clonal distribution pattern of the gene suggests that *ssa* may have been acquired by individual clones via horizontal transfer and recombination. The

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Baylor College of Medicine, DeBakey Bldg. Rm. M929, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-6054. Fax: (713) 798-3700.

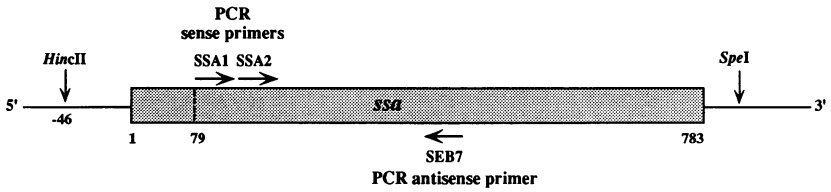

FIG. 1. PCR amplification and cloning strategy of *ssa* from Weller strain genomic DNA. The 5′ half of *ssa* was amplified with nondegenerate, nonoverlapping oligonucleotides designed from SSA N-terminal protein sequence information (SSA1 and SSA2) paired with an antisense primer specific for SEB (SEB7). An approximately 340-bp PCR product amplified by the SSA1-SEB7 primer pair was used to screen a Weller strain subgenomic phage library for the full-length SSA gene. The *ssa* open reading frame was subcloned into the pBluescript KS$^-$ expression vector by use of an upstream *Hin*cII site and a downstream *Spe*I site. Numbers correspond to nucleotides in Fig. 2.

presence of *ssa* in isolates of the ET 2-M3 clonal lineage causing contemporary invasive disease episodes, but not in organisms of this clone cultured from patients in the 1910s and 1920s, is consistent with a recent acquisition of *ssa* by the ET 2-M3 clonal lineage of *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. pyogenes* ET 2-M3 strain Weller (also designated DLS88002) (39) was the source strain for the SSA gene. Strain Gall (also designated DLS89026) (39), serotype M1, was used as a negative control in Southern blot and PCR screenings. These strains were recovered from patients with fatal TSLS and were kindly provided by D. L. Stevens, Veterans Administration Hospital, Boise, Idaho. The panel of serotyped organisms was obtained from M. Kehoe and is the strain set used by the Streptococcal Reference Center at the Central Public Health Laboratory, London, United Kingdom. ET 2-M3 strains are from the *S. pyogenes* collection of J.M.M. Contemporary ET 2-M3 isolates were isolated from patients with severe streptococcal disease in the United States between 1986 and 1990 (40). ET 2-M3 strains isolated between 1969 and 1979 and between 1940 and 1941 are from scarlet fever epidemics in eastern Germany and in Ottawa, Canada, respectively (42). The historical designations of invasive ET 2-M3 isolates recovered early in this century are as follows: the 1910 strain (594; scarlet fever isolate), the 1920 strain (C203; scarlet fever isolate), and the 1937 strain (D-58; puerperal septicemia isolate) (41). *S. pyogenes* cultures were grown in brain heart infusion medium (Difco, Detroit, Mich.) without shaking at 37°C in a humidified incubator supplemented with 5% CO_2. XL-1 Blue *Escherichia coli* (Stratagene, La Jolla, Calif.) clones were cultured at 37°C with shaking in broth (16 g of Bacto Tryptone, 10 g of yeast extract, 5 g of sodium chloride [per liter]) with 100 μg of ampicillin per ml. pBluescript (Stratagene) plasmids carried by individual XL-1 Blue strains included pMV7, a pBluescript SK$^-$ derivative encoding SEB; pKR1 and pKR2, pBluescript SK$^-$ derivatives encoding the 5′ 338 bp and 305 bp, respectively, of SSA; and pKR4, a pBluescript SK$^-$ derivative containing the entire *ssa* open reading frame.

Preparation of streptococcal genomic DNA. Bacteria from a 1-liter overnight culture of strain Weller were harvested by centrifugation, resuspended in 10 mM Tris–1 mM EDTA (TE) (pH 8.0) containing 100 μg of mutanolysin (Sigma, St. Louis, Mo.) and 5 mg of lysozyme (Sigma) per ml, and incubated at 37°C for 2 h. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1.0%, and the suspension was heated to 65°C for 30 min. Cell debris was sedimented by centrifugation, and proteinase K (0.3 mg/ml) was added to the supernatant. After incubation for 2 h at 37°C, the sample was extracted with phenol-chloroform and precipitated with sodium acetate and ethanol for 2 h at −20°C. Nucleic acids were then pelleted by centrifugation, washed once with 70% ethanol, and resuspended in 2 ml of TE. DNA was purified by dye-buoyant density ultracentrifugation in a TLA100.3 rotor (Beckman, Palo Alto, Calif.) at 70,000 rpm for 22 h at 22°C. Ethidium bromide was removed by repeated extraction with isoamyl alcohol. DNA was precipitated with ethanol and resuspended in 400 μl of H_2O.

PCR amplification and cloning of the 5′ half of *ssa* from Weller genomic DNA. Nondegenerate, nonoverlapping oligonucleotides (SSA1, 5′-AGTCAACCAGATCCTACGCCAG AACAATTGAA-3′; SSA2, 5′-AAATCGAGTCAATTTACA GGAGTTATGGCC-3′) were designed on the basis of the SSA N-terminal protein sequence (39) with a bias toward SEB codon usage (29). We hypothesized that SSA may retain homology to SEB in regions further downstream from the 24 N-terminal residues, especially in regions relatively conserved among all known staphylococcal and streptococcal superantigens. In order to amplify *ssa* from Weller genomic DNA with PCR, we paired each SSA oligonucleotide with an oligonucleotide (SEB7, residues 658 to 675 [5]) specific for a region in SEB immediately downstream of the disulfide loop (Fig. 1). Weller genomic DNA (200 ng) was combined with 50 pmol each of sense (SSA1 or SSA2) and antisense (SEB7) primers, a 200 μM concentration of each deoxynucleoside triphosphate, and 10 μl of 10× *Pfu* polymerase buffer 1 (Stratagene) in a total volume of 100 μl. Reaction mixtures were overlaid with 100 μl of mineral oil and denatured at 95°C for 7 min before *Pfu* polymerase (2.5 units) (Stratagene) was added. PCR conditions were as follows: 1 min at 95°C, 2 min at 37°C, and 3 min at 72°C for 25 cycles in a thermocycler (Perkin-Elmer Corp., Norwalk, Conn.). Combinations of SSA1 or SSA2 with SEB7 specifically amplified products of approximately 340 or 310 bp, respectively, from strain Weller genomic DNA, but not from strain Gall DNA, which does not produce SSA. PCR products were ligated to the pBluescript SK$^-$ vector to make pKR1 and pKR2, which were used to transform XL-1 Blue *E. coli*. Nucleotide sequence analysis of pKR1 and pKR2 inserts predicted amino acid sequences identical to that determined by N-terminal protein sequencing of native SSA from strain Weller *S. pyogenes* (39).

Subgenomic library construction and screening. Both Weller PCR products hybridized to a 7.4-kb *Eco*RI fragment of Weller genomic DNA by Southern blot analysis. Using the 340-bp PCR product as a probe, we then isolated the entire *ssa* gene within a 7.4-kb *Eco*RI fragment from a Weller strain subgenomic library. To construct the subgenomic library, Weller genomic DNA was digested to completion with *Eco*RI and size fractionated on a 0.8% agarose gel. The region containing 6.5- to 9.0-kb fragments was excised, and the DNA

was electroeluted. Eluted DNA was concentrated and purified with an Elutip-D column (Schleicher & Schuell, Keene, N.H.), ethanol precipitated, and resolubilized in 10 μl of H_2O. This fraction of genomic DNA was then ligated to the *Eco*RI-digested, alkaline phosphatase-treated arms of the Lambda-ZAP II vector (Stratagene) according to the manufacturer's specifications. Recombinants were packaged with the Gigapack II Gold Packaging Extract kit (Stratagene). The phage library was screened by hybridization according to the support protocol provided with the Lambda-ZAP II vector, by means of the 340-bp PCR product labeled by random priming (Prime-It; Stratagene) with [α-^{32}P]dATP (DuPont-NEN, Boston, Mass.) as a probe. Phage were eluted from positive plaques (12 of 1,000) and converted to plasmid form (pBluescript) for nucleotide sequencing by an in vivo excision process.

DNA sequencing. For sequencing of recombinant *ssa*-containing clones, double-stranded plasmid DNA was alkali denatured and sequenced with dideoxy termination sequencing Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) as detailed in the manufacturer's instructions. The SSA gene was sequenced four times in both orientations.

Screening of group A streptococcal strains by PCR. PCR reactions were done as described above with 200 ng of streptococcal genomic DNA and 50 pmol each of sense and antisense primers (*ssa* internal primers corresponding to nucleotides 82 to 98 and 757 to 772; Fig. 2). Two primers specific for the streptococcal pyrogenic exotoxin B gene (*speB*), a gene present in virtually all extant group A streptococcal strains (22), were used as internal positive controls for each sample (41). PCR conditions were as follows: 1 min at 95°C, 2 min at 55°C, and 3 min at 72°C for 25 cycles. Portions (10 μl) of reaction products were electrophoresed on a 1.0% agarose gel and visualized by staining with ethidium bromide.

Southern blot analysis. Streptococcal genomic DNA was digested to completion with *Eco*RI, fractionated on a 0.8% agarose gel, transferred to Hybond N (Amersham, Arlington Heights, Ill.) with a pressure blotter (Posi-Blot; Stratagene), and fixed to the membrane by UV cross-linking with a Stratalinker (Stratagene). The blot was then prehybridized in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–5× Denhardt's solution–1% Sarkosyl–0.002% salmon sperm DNA for a minimum of 4 h at 37°C. Blots were hybridized for 20 h at 65°C with the full-length SSA gene labeled by random priming as a probe. Blots were washed in 2× SSPE–0.1% SDS four times for 15 min each time at 25°C and in 0.1× SSPE–0.1% SDS twice for 10 min each time at 65°C.

Subcloning and expression of recombinant SSA. The nucleotide sequence encoding the mature form of SSA was PCR amplified from Weller genomic DNA with flanking primers and digested with *Hinc*II and *Spe*I, which cut 46 bp upstream and 33 bp downstream, respectively, of the *ssa* open reading frame (Fig. 1). This fragment was ligated to the pBluescript II KS$^-$ expression vector (Stratagene) to make pKR4. An XL-1 Blue *E. coli* strain carrying pKR4 was grown to an optical density at 600 nm of 1.0 and induced to express SSA by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.1 mM. After further incubation at 37°C with shaking for 3 h, bacteria were harvested by centrifugation and resuspended in TE, pH 8.0. An aliquot of cells was then mixed with an equal volume of SDS sample buffer, and the whole cell lysate was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and SSA immunoblot analysis. Lysates of *E. coli* strains carrying pMV7 or pBluescript, containing *seb* or no insert in the multicloning cassette, respectively, were processed in parallel as positive and negative controls. Uninduced whole cell lysates were also examined.

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                -35                                  -10
GGTGTAGAATTGAGGTAATTGGGGAGATGAAAAGTTAACGTTATAATCTATATAACGTT

                (SD)          start
TTAAATATATAAGAGGTAGCCAA ATG AAT AAA AGA ATA AGA ATA CTC GTT
                          M   N   K   R   I   R   I   L   V

 28 GTA GCA TGT GTA GTT TTT TGT GCA CAA TTA TTA TCG ATT AGT GTT
     V   A   C   V   V   F   C   A   Q   L   L   S   I   S   V

 73 TTT GCA AGT AGT CAG CCT GAC CCT ACT CCA GAA CAA TTA AAC AAA
     F   A  ⇑S   S   Q   P   D   P   T   P   E   Q   L   N   K

118 TCT AGC CAA TTT ACT GGT GTT ATG GGT AAT TTG AGA TGT TTA TAT
     S   S   Q   F   T   G   V   M   G   N   L   R   C   L   Y

163 GAT AAC CAT TTT GTA GAA GGG ACT AAT GTA AGA TCC ACA GGT CAG
     D   N   H   F   V   E   G   T   N   V   R   S   T   G   Q

208 CTT TTA CAG CAT GAT TTG ATT TTT CCC ATT AAG GAT TTG AAA CTT
     L   L   Q   H   D   L   I   F   P   I   K   D   L   K   L

253 AAA AAC TAT GAT TCA GTA AAA ACA GAG TTC AAT AGT AAA GAT TTA
     K   N   Y   D   S   V   K   T   E   F   N   S   K   D   L

298 GCT GCA AAA TAT AAA AAT AAA GAT GTA GAT ATT TTT GGT TCC AAT
     A   A   K   Y   K   N   K   D   V   D   I   F   G   S   N

343 TAC TAC TAT AAC TGC TAT TAT TCG GAA GGA AAT AGT TGT AAA AAT
     Y   Y   Y   N   C   Y   Y   S   E   G   N   S   C   K   N

388 GCC AAA AAA ACT TGT ATG TAC GGA GGT GTT ACT GAG CAC CAT AGA
     A   K   K   T   C   M   Y   G   G   V   T   E   H   H   R

433 AAT CAA ATT GAA GGT AAG TTC CCG AAT ATT ACA GTA AAA GTT TAT
     N   Q   I   E   G   K   F   P   N   I   T   V   K   V   Y

478 GAA GAT AAT GAA AAT ATA CTT TCG TTT GAT ATT ACA ACT AAT AAA
     E   D   N   E   N   I   L   S   F   D   I   T   T   N   K

523 AAA CAA GTC ACT GTT CAA GAA TTA GAT TGT AAA ACC AGA AAA ATA
     K   Q   V   T   V   Q   E   L   D   C   K   T   R   K   I

568 TTA GTG TCT CGA AAA AAT TTG TAT GAG TTC AAT AAT TCT CCG TAC
     L   V   S   R   K   N   L   Y   E   F   N   N   S   P   Y

613 GAG ACA GGC TAT ATT AAA TTT ATA GAG AGC TCA GGA GAC AGT TTT
     E   T   G   T   I   K   F   I   E   S   S   G   D   S   F

658 TGG TAC GAT ATG ATG CCC GCA CCT GGA GCA ATA TTT GAT CAG TCT
     W   Y   D   M   M   P   A   P   G   A   I   F   D   Q   S

703 AAA TAC TTA ATG CTA TAT AAC GAT AAT AAG ACA GTG AGC TCT TCA
     K   Y   L   M   L   Y   N   D   N   K   T   V   S   S   S

748 GCT ATA GCT ATA GAG GTT CAC CTT ACC AAA AAA TAA AAGAAAATAAC
     A   I   A   I   E   V   H   L   T   K   K  end

    TTTATGTATCAAATCCATGATACTAGTTAAATTCAAAATGATTGATATTAAAAAAGTTA
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FIG. 2. Nucleotide and deduced amino acid sequences of *ssa* and SSA, respectively. Regions with homology to −10 and −35 bacterial promoter consensus sequences and the Shine-Dalgarno (SD) ribosome binding site are underlined. The *ssa* open reading frame is 783 bp long and encodes a predicted 260-amino-acid protein. The cleavage site of the signal peptide between amino acids 26 and 27, as determined by N-terminal sequencing of mature SSA (38), is indicated by a vertical arrow.

Computer analysis. Protein sequences were aligned with the program CLUSTAL V (23). Percentage scores of identity take into consideration the number and size of the gaps introduced when aligning each protein with SSA to yield maximum homology. A 50% majority rule consensus tree based on protein sequence identity was generated with PAUP 3.0q (52) and the bootstrap method (15) applied with heuristic search and nearest-neighbor interchange branch swapping without weighting conserved or functionally important regions.

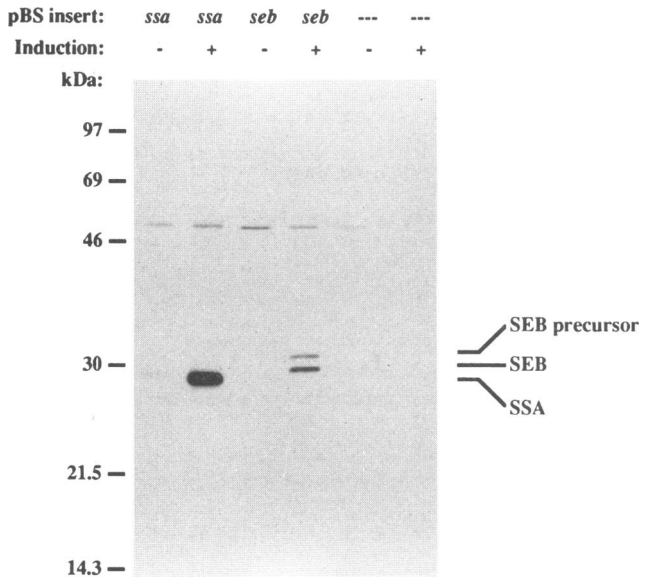

FIG. 3. *ssa* encodes a protein that migrates at ~28 kDa and is immunoreactive with anti-SSA immunoglobulin G. Whole-cell lysates from XL-1 Blue *E. coli* transformed with pBluescript II SK$^-$ alone (lanes 5 and 6) or with pBluescript containing either the *ssa* (lanes 1 and 2) or the *seb* (lanes 3 and 4) open reading frame were screened for toxin production with anti-SSA immunoglobulin G purified from an antipeptide antiserum that was raised against the first 16 residues of native SSA (38). Both the SEB precursor and mature SEB were present in induced *seb* transformant lysates (lane 4), but lysates from IPTG-induced *ssa* transformants had only the mature form of SSA (lane 2). Precursor SSA is shown as a faint band in the uninduced lysates (lane 1). Induced and uninduced cell lysates from transformants with and without superantigen inserts (all lanes) had an ~52 kDa immunoreactive protein.

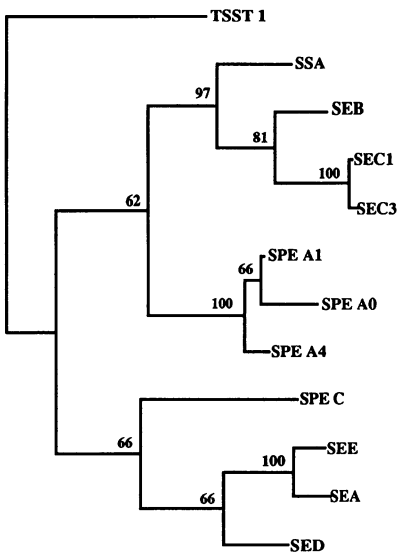

FIG. 4. Phylogenetic analysis of staphylococcal and streptococcal superantigen toxins. Amino acid sequences were obtained from the data bases GenBank and EMBL (SEA, M18970; SEB, M11118; SEC1, X05815; SEC3, M28364; SED, M28521; SEE, M21319; SPE A0, X03929; SPE A1, X61560; SPE A 4, X61573; SPE C, M35514; and TSST-1, J02615) and aligned with the program CLUSTAL V (23). A 50% majority rule consensus tree was generated with the computer program PAUP 3.0q (52) and the bootstrap method (15) applied with heuristic search and nearest-neighbor interchange branch swapping. The numbers adjacent to nodes represent the proportions of 2,000 bootstrap trees that contained the taxa to the right as a monophyletic group.

Nucleotide sequence accession number. The DNA sequence for *ssa* has been deposited in GenBank under accession number L29565.

RESULTS

Nucleotide sequence of the *ssa* gene. The nucleotide sequence of *ssa* is shown in Fig. 2. The open reading frame is 783 bp, including translation initiation and termination codons. A purine-rich region located 7 bp upstream from the start codon is complementary to the 3′ end of *Bacillus subtilis* 16S rRNA and is a putative ribosome binding site (49). Regions with significant homology to bacterial consensus −10 (TATAAT) and −35 (TTGACA) promoter regions (46) are underlined. The open reading frame encodes a predicted 260-amino-acid protein with a molecular weight of 29,771 (Fig. 2). Amino acid sequence analysis of SSA previously identified residue S-27 as the N terminus of the mature protein (39). The first 26 residues encoded by *ssa* form a leader sequence with basic and hydrophobic domains that targets the mature protein with a molecular weight of 26,892 for secretion. The presumed cleavage site of the signal sequence is designated by an arrow (Fig. 2).

Expression of recombinant SSA. SDS-PAGE and immunoblot analysis of whole-cell lysates from an *E. coli* clone carrying a plasmid with the *ssa* open reading frame confirmed that *ssa* encodes a protein that migrates at ~28 kDa and is immunoreactive with anti-SSA immunoglobulin G (Fig. 3). Because this antiserum was raised against an N-terminal peptide of SSA (39), it is also immunoreactive with SEB, which has 62.5% identity to SSA in this region (see Fig. 5).

Both the precursor and the mature form of SEB were present in induced cell lysates. However, only the mature form of SSA was observed, perhaps because SSA precursor was more rapidly and/or completely processed than SEB precursor. A faint 30-kDa band is seen in the uninduced lysates of *ssa* transformants, suggesting that SSA precursor synthesis occurs at low levels without induction. The expression of SSA precursor in lysates of uninduced cultures may be due to the retention of native promoter elements in the 5′ flanking region of the open reading frame (Fig. 2). The *seb* transformant lacks upstream noncoding regulatory sequences and did not express SEB without induction. Analysis of periplasmic fractions from induced cultures indicated that only the mature forms of SSA and SEB are secreted (data not shown).

An immunoreactive 50-kDa protein is seen in induced and uninduced samples with and without superantigen-encoding inserts and most likely represents a cross-reactive *E. coli* product.

Relatedness of SSA to other bacterial superantigens. Amino acid sequence alignment of SSA with other bacterial superantigens determined that SSA is 60.2 and 59.2% identical to SEB and SEC3, respectively, but only 49% identical to SPE A (see Fig. 5). SSA shares between 27 and 32% of its sequence with the staphylococcal superantigens SEA, SED, and SEE. Minimal homology is seen in comparisons of SSA with the more divergent toxins SPE C (22.4%) and TSST-1 (20.8%).

A phylogenetic tree based on protein sequence identity illustrates the relatedness of the staphylococcal and streptococcal superantigens (Fig. 4). Interestingly, the three alleles of

```
                10         20         30         40         50
SSA:  SSQPDPTPEQ LNKSSQFTGV MGNLRCLYDN HFVEGTNVRS TGQLLQHDLI
SEB:  E-----K-DE -G---K---L -E-MKV---D NH-SAI--K- ID-F-YF---
SEC3: E-----M-DD -H---E---T ---MKY---D -Y-SA-K-K- VDFK-A----
SPEA:   -Q--D-S- -HR--.LVKN LQ-ITF--EG DP-THE--K- VD---S----

                60         70         80         90        100
SSA:  FPIKDLKLKN YDSVKTEFNS KDLAAKYKNK DVDIFGSNYY YNCYYSEGNS
SEB:  YS---T--G- --N-RV--KN ----D---D- Y--V--A--- -Q--F-KKTN
SEC3: YN-S-K---- --K----LLN E---K---DE V--VY----- V---FS-KD-
SPEA: YNVSGP...- --KL---LKN QEM-TLF-D- N---Y-VE-- HL--LC-NAE

               105        115        124        134        144
SSA:  CKNA.....K KTCMYGGVTE HHRNQIE.GK FPNITVKVYE DNENILSFDI
SEB:  DI-SHQTDKR ---------- -NG--LD..- YRS---R-F- -GK-L----V
SEC3: VG-VTGG--- ------I-K -EG-HFDN-N LQ-VL-R--- NKR-TI--EV
SPEA: .........R SA-I-----N -EF-HL-IP- ..K-V---SI -GIQS-----

               154        164        174        184        194
SSA:  TTNKKQVTVQ ELDCKTRKIL VSRKNLYEFN NSPYETGYIK FIESSGDSFW
SEB:  Q----K--A- ---TL--HY- -KN-K----- ---------- ---NE.N---
SEC3: Q-D--S--A- ---I-A-NF- INK------- S--------- ---NN-NT--
SPEA: E----M--A- ---Y-V--Y- TDN-Q--TNG P-K------- --PKNKE---

               204        214        224        234
SSA:  YDMMPAPGAI FDQSKYLMLY NDNKTVSSSA IAIEVHLTKK     234 aa
SEB:  --------DK --------M- ----M-D-KD VK---Y--T-  KK 239 aa
SEC3: --------DK --------M- ------D-KS VK------T-  NG 239 aa
SPEA: --FF-E-E.. -T------I- K--E-LD-NT SQI            221 aa
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FIG. 5. Several domains shared among bacterial superantigens are conserved in SSA. The alignment of the amino acid sequence of the mature form of SSA with those of SEB, SEC3, and SPE A was constructed with the CLUSTAL V algorithm (23). Regions of identity are indicated by dashed lines, and each period represents a gap of one amino acid. The numbers above the sequences correspond to the amino acid residues of the mature form of SSA. Boxes indicate conserved cysteine residues at positions 93 and 108.

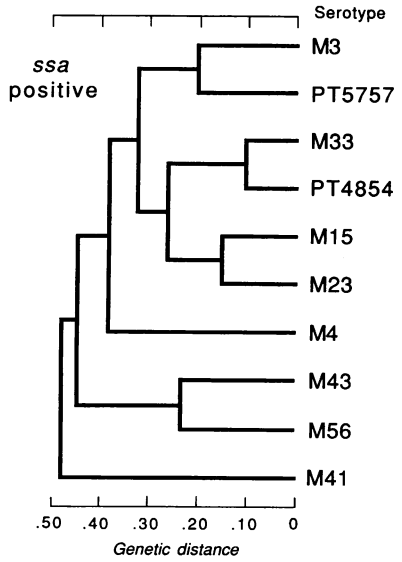

FIG. 6. The *ssa* gene is distributed among ten phylogenetically diverse group A streptococcal lineages. The dendrogram shows estimates of genetic relationships among the 10 distinct *ssa*-positive clonal lineages of *S. pyogenes*, based on multilocus enzyme electrophoresis allele profiles at 12 enzyme loci. Each lineage is designated by an M serotype or a provisional M type number.

SPE A shown diverge from SEB, SEC1, and SEC3 earlier than does SSA. While 97% of all bootstrap trees generated contained SSA, SEB, and the SEC proteins as a monophyletic group, only 62% also included SPE A (Fig. 4).

Prediction of functionally important amino acid residues in SSA. All of the staphylococcal and streptococcal superantigens except TSST-1 have a conserved central disulfide loop that varies in length from 9 to 19 intervening amino acid residues (35). Mutational and biochemical studies have shown that the disulfide loop is important for superantigen-mediated T-cell proliferation (20). The amino acid sequence within the disulfide loop is not conserved among superantigens, suggesting that it is instead the tertiary structure of the loop and its placement within the protein that are critical for mitogenicity. Alignment of the predicted amino acid sequences of SSA, SEB, SEC3, and SPE A (Fig. 5) shows that SSA is similar in length to the other toxins and has two conserved central cysteine residues, C93 and C108, that may form a disulfide loop with 14 intervening residues.

SSA also shares with other bacterial superantigens a large region between amino acid residues 107 and 162 that is homologous to the C-terminal end of both the mouse (positions 121 to 169) and the human (positions 168 to 216) class II invariant chains, proteins that associate with nascent MHC class II molecules (35).

Distribution of *ssa* and SSA production among group A streptococci. In order to assess the prevalence of *ssa* among group A streptococci, we screened 138 isolates of *S. pyogenes*, representing 65 different M protein serotypes and 15 nontypeable strains, by PCR with primers specific for the 5′ and 3′ termini of *ssa*. Each strain was also analyzed by Southern blot analysis with a full-length *ssa* probe, in the event that a strain might possess only a fragment of the gene or have allelic polymorphism at the primer sites. All strains that were negative for *ssa* by PCR were also negative by Southern blot analysis (data not shown).

ssa was present in only 10 (12.5%) of the 80 clonal lineages tested, including one of two highly pathogenic clones responsible for the majority of contemporary severe disease episodes caused by *S. pyogenes* (40). The ET 2-M3 lineage, a clone associated with rheumatic fever, severe invasive disease, and TSLS, had the SSA gene. However, we did not identify *ssa* in strains of the virulent ET 1-M1 clone. Of the other nine *ssa*-positive lineages, T4 (M4) has been associated with group A streptococcal pharyngitis and scarlet-fever-like skin lesions (43). Clones expressing M15, M23, M33, M41, M43, M56, or provisional M types PT5757 and PT4854 do not commonly cause invasive infections and are rarely isolated from patients with other streptococcal diseases.

A dendrogram estimating overall chromosomal relatedness of the *ssa*-positive strains, based on multilocus enzyme electrophoresis (40, 48), is shown in Fig. 6. These phylogenetic lineages are in many cases highly divergent in chromosomal character and on average are not more related to each other than they are to strains of other M protein serotypes that do not encode SSA (data not shown).

To determine if SSA production correlated with the presence of the gene, we screened culture supernatants of strains from each *ssa*-positive lineage. Immunoblot analysis found all strains with *ssa* to express mature SSA (data not shown).

Temporal variation of *ssa* occurrence in the ET 2-M3 clonal lineage. Genomic DNA from a panel of 52 ET 2-M3 strains was screened by PCR and Southern blot analysis for *ssa*. All 38 contemporary (1969 to 1990) invasive ET 2-M3 disease isolates and 11 strains recovered from patients with scarlet fever between 1940 and 1941 had *ssa*. One ET 2-M3 puerperal

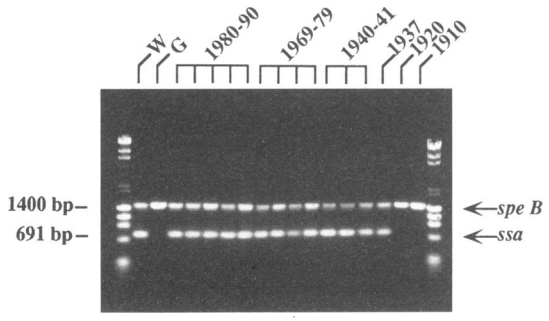

FIG. 7. The ET 2-M3 clonal lineage exhibits temporal variation in *ssa* association. Genomic DNA samples from 15 representative ET 2-M3 isolates (from a panel of 52) were PCR amplified with primers specific for *ssa*. A separate pair of primers specific for *speB* was used as a positive control. The SSA gene is shown in 13 representative ET 2-M3 isolates from the following time periods, clinical conditions, and geographic localities: 1980 to 1990, five severe invasive disease or TSLS isolates, United States; 1969 to 1979, four scarlet fever isolates, eastern Germany; 1940 to 1941, three scarlet fever isolates, Ottawa, Canada; 1937, one puerperal septicemia isolate, United States. Only 2 of the 52 ET 2-M3 strains screened were *ssa* negative: one scarlet fever isolate from 1920 and one scarlet fever isolate from 1910. Genomic DNA samples from Weller (W) and Gall (G) strains were used as positive and negative controls, respectively, for *ssa*. All ET 2-M3 *S. pyogenes* isolates screened were *speB* positive.

septicemia isolate from 1937 was *ssa* positive. Surprisingly, two older scarlet fever isolates of the same clonal lineage cultured in the 1910s and 1920s did not have the *ssa* gene (Fig. 7).

DISCUSSION

Bacterial superantigens are potent stimulators of T-cell proliferation and lymphokine secretion. This family of proteins includes several extracellular toxins elaborated by *S. pyogenes* and *S. aureus*, most notably streptococcal pyrogenic exotoxins A and C, staphylococcal enterotoxins A through F, and staphylococcal TSST-1. These toxins have considerable amino acid homology and elicit similar biological responses. In addition to Vβ-specific T-cell stimulation, bacterial superantigens induce fever, mediate T-cell-dependent immunosuppression of immunoglobulin secretion, enhance susceptibility to endotoxin shock, and trigger cytokine release from MHC class II-positive cells (3). Superantigens are thought to play a critical role in the pathogenesis of staphylococcal toxic shock syndrome (11) and perhaps of Kawasaki disease (1, 34) and several autoimmune disorders, including rheumatoid arthritis (16, 44) and acute rheumatic fever (19).

Because the immunomodulatory effects of superantigens may play a role in invasive and autoimmune diseases, it is important to understand the molecular basis of their interactions with immune system components. Functional analysis of SEB mutants (32) and solution of the crystal structure of SEB (51) have identified amino acid residues important for MHC class II binding and subsequent interaction with specific Vβ regions of the T-cell receptor. Our studies show that SSA is 60.2% identical to the staphylococcal superantigen SEB. On the presumption that the crystal structures of SSA and SEB are isomorphic, it may be possible to predict functionally important regions in the SSA molecule.

SSA has the same MHC class II isotype preferences as SEB, stimulating T-cell proliferation in the presence of HLA-DR, HLA-DQ, and H-2 IE^d (39). The N-terminal regions in SEB identified in functional studies as important in MHC class II binding include amino acid residues 14 to 23 and 41 to 53 (32). Our analysis shows that some of these amino acid residues are conserved in SSA. For example, within the first region, 7 of 10 residues are identical in SEB and SSA, including residue N-23, which is important in both MHC binding and Vβ interactions (32). Conversely, residue F-44, identified as essential for high-affinity DR1 binding in SEB functional studies (32), is replaced by L-44 in SSA. Interestingly, the substitution F-44→L in SEB reduced binding to DR1 by 1,000-fold and hybridoma interleukin 2 release by 100-fold (32). The fact that SSA stimulates T-cell proliferation in the presence of DR1 suggests that SSA binds to the DR1 molecule with altered affinity and/or conformation, or at an entirely separate location, compared to SEB.

SSA stimulates T cells bearing Vβ1, Vβ3, Vβ5.2, and Vβ15 (39). SSA shares some of these Vβ specificities with SEB, such as Vβ3 and Vβ15 (39), and it is therefore noteworthy that residues N-60 and Y-61, which are essential for certain SEB-Vβ associations (32), are conserved in SSA. Additional regions in SEB important for T-cell receptor binding include amino acid residues from both N-terminal and C-terminal portions of the primary sequence (51). For example, C-terminal residues D-206 and K-207 appear to define the outer rim of a putative SEB T-cell receptor-binding cavity (51). Independent functional studies have recently corroborated the importance of the corresponding residues in SEA and SEE for specific Vβ interactions (27, 28, 38). Interestingly, SSA differs from SEB at only three amino acid residues between 195 and 221 (residues 193 and 218 in SSA): D-206→A, K-207→I, and M-216→L (positions 203, 204, and 213, respectively, in SSA). The changes specific to SSA at positions 203 and 204 substitute two nonpolar amino acids for two polar amino acids. The increase in hydrophobicity may alter the local conformation of the protein and vary the Vβ specificity of SSA with respect to SEB. These amino acid changes may explain why SSA stimulates human Vβ1 but SEB does not and why SEB stimulates human Vβ12, Vβ14, Vβ17, and Vβ20 but SSA does not (39).

The similarity of SSA, made by *S. pyogenes*, to SEB, made by *S. aureus*, suggests that their structural genes have a common precursor. It has been proposed that staphylococcal and streptococcal superantigen genes arose from a shared ancestral gene before the evolutionary divergence of the two organisms (17). This hypothesis accounts for the sequence homology seen among the superantigens of both genera. A common ancestor organism is consistent with the observation that both staphylococci and streptococci are gram-positive cocci, have an A+T content of approximately 70%, and express several analogous gene products (17).

The presence of several superantigen genes on mobile elements supports a role for horizontal transfer and recombination in their dissemination. The genes encoding SPE A, SPE C, SEA, and SEE reside on bacteriophages (3, 4, 18, 54), and plasmid-borne toxin genes include *seb* and *sed* (3). Molecular population genetic methods such as comparative sequencing and phylogenetic analyses of toxin distribution have provided strong evidence for the lateral transfer of *speA* and *speC* between distinct streptococcal clonal lineages (33, 40). In this regard, it will be important to determine if *ssa* resides on a mobile genetic element.

Our data show that *ssa* is distributed throughout the species among 10 distinct clonal lineages. These lineages are in many instances phylogenetically divergent in overall chromosomal character and therefore have not shared a recent common ancestor. Two hypotheses account for the pattern of *ssa* distribution observed among group A streptococci. The first hypothesis invokes *ssa* as an ancestral condition in *S. pyogenes*

and proposes that each *ssa*-positive lineage inherited the SSA gene from a common ancestor strain. Alternatively, *ssa* may have been acquired independently by several different phylogenetic lineages, perhaps by bacteriophage-mediated transduction and recombination. Although we cannot test these two hypotheses at present, it is noteworthy that other bacterial superantigens are bacteriophage encoded and capable of horizontal transfer. Additionally, support for *ssa* as an ancestral condition would need to account for the loss of the gene in the majority of contemporary *S. pyogenes* lineages.

Analysis of the ET 2-M3 clonal lineage found evidence for temporal variation in *ssa* distribution. We identified *ssa* in all 38 contemporary ET 2-M3 invasive disease isolates screened, 11 strains from the 1940s, and one isolate from 1937, but two older ET 2-M3 scarlet fever isolates recovered in the 1910s and 1920s did not carry the gene. Because of the limited availability of streptococcal strains from early in this century, the two older isolates examined here may not be fully representative of the M3 strains causing severe disease at that time. It is also possible that these strains originally had *ssa* but lost the gene after repeated culturing. However, strains with bacteriophage-encoded *speA* retain the toxin gene for at least 20 passages in vitro (22), suggesting that this gene, and perhaps those of related toxins, is relatively stable. Our observations are consistent with a recent acquisition of *ssa* by the ET 2-M3 lineage.

Temporal variations in the *speA* (41) and *speC* (33) genes have been described over intervals of time similar to those observed here. Both *speA* and *speC* exhibit a temporally dependent allelic diversity, and new waves of scarlet fever have been associated with an increase in frequency of *S. pyogenes* clones carrying variant *speA* alleles (42). The acquisition of new virulence factors or mutations in loci to generate new toxin variants may be involved in temporal variation in the frequency and severity of *S. pyogenes*-mediated disease (42). Inasmuch as other streptococcal superantigen genes display allelic variation among distinct phylogenetic lineages, the variation in virulence of *ssa*-positive bacterial clones may be explained by the occurrence of clone-virulence factor allele combinations. It would be of interest to determine if allelic variation exists in the SSA gene, and studies are under way to examine this possibility.

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