INFECTION AND IMMUNITY, Sept. 1997, p. 3600–3605 Vol. 65, No. 9
0019-9567/97/$04.00+0

Comparison of the Sequences and Functions of *Streptococcus equi* M-Like Proteins SeM and SzPSe

JOHN F. TIMONEY,* SERGEY C. ARTIUSHIN, AND JEFFREY S. BOSCHWITZ

Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546-0099

Received 5 February 1997/Returned for modification 30 April 1997/Accepted 16 June 1997

***Streptococcus equi* (*Streptococcus equi* subsp. *equi*), a Lancefield group C streptococcus, causes strangles, a highly contagious purulent lymphadenitis and pharyngitis of members of the family Equidae. The antiphagocytic 58-kDa M-like protein SeM is a major virulence factor and protective antigen. The amino acid sequence and structure of SeM has been determined and compared to that of a second, 40-kDa M-like protein (SzPSe) of *S. equi* and to those of other streptococcal proteins. Both SeM and SzPSe are mainly alpha-helical fibrillar molecules with no homology other than that between their signal and membrane anchor sequences and are only distantly related to other streptococcal M and M-like proteins. The sequence of *SzPSe* indicates that it is an allele of *SzP* that encodes the variable protective M-like and typing antigens of *S. zooepidemicus* (*S. equi* subsp. *zooepidemicus*). SeM is opsonogenic for *S. equi* but not for the closely related *S. zooepidemicus*, whereas SzPSe is strongly opsonogenic for *S. zooepidemicus* but not for *S. equi*. Both proteins bind equine fibrinogen. SeM and SzPSe proteins from temporally and geographically separated isolates of *S. equi* are identical in size. The results taken together support previous evidence that *S. equi* is a clonal pathogen originating from an ancestral strain of *S. zooepidemicus*. We postulate that acquisition of SeM synthesis was a key element in the success of the clone because of its effect in enhancing resistance to phagocytosis and because protective immunity entails a requirement for SeM-specific antibody.**

Streptococcus equi, a Lancefield group C streptococcus, causes strangles, a highly contagious disease of the nasopharynx and draining lymph nodes of members of the family Equidae. The 58-kDa antiphagocytic M-like protein SeM is a major virulence factor and protective antigen and functions by limiting deposition of C3b on the bacterial surface and by directly binding fibrinogen (4, 5). A second M-like protein (SzPSe) that is antigenically cross-reactive with the hypervariable M-like (SzP) proteins of the closely related *Streptococcus zooepidemicus* (*S. equi* subsp. *zooepidemicus*) is also produced (25). Isolates of *S. equi* are antigenically and genetically similar, and protective immunity is species specific (1, 7, 15). Thus, immunization with *S. zooepidemicus* does not protect against challenge by *S. equi*, although equine isolates of these two streptococci show better than 92% DNA homology (13, 24). Recent multilocus enzyme electrophoresis (MEE) studies have confirmed the close genetic relationship of *S. equi* and *S. zooepidemicus* and have indicated that the former is a clone derived from the more genetically diverse *S. zooepidemicus* (12). This finding has resulted in the recommendation that the organism currently designated *S. equi* subsp. *equi* be reclassified as a biovar of *S. zooepidemicus* (12).

The designation M-like protein is applied to streptococcal fibrillar surface proteins with a variety of ligand activities but with common structural features, including signal and cell wall-associated sequences, variable N-terminal regions, conserved central C repeats, and proline-rich C-terminal domains (19). In a few M-like proteins (e.g., FcrA and Mrp), the C repeats are replaced by an A repeat region (16). It is generally agreed that the term M protein should be reserved for those M-like proteins that induce opsonic antibodies specific for the strain from which the protein was derived (6). M proteins with these characteristics were first described for the Lancefield group A streptococci and subsequently have been found on groups C and G streptococci (3, 20). The protective M-like protein SzP of *S. zooepidemicus* is a 40.1-kDa, acid-resistant protein that elicits serum opsonic and protective responses in mice and serum opsonic responses in horses (23, 26). This protein is antigenically variable, extractable with hot acid or mutanolysin, and appears to be the typing antigen of the Moore and Bryans serovars (15, 24). SzP proteins, although M-like, do not have A, B, or C repeat domains as seen in the group A M-like proteins to which they appear to be only distantly related. A homolog of SzP, SzPSe, also occurs on *S. equi* cells, but its role in protective immune responses to *S. equi* has not been determined (25, 27).

The objectives of this study were to obtain the amino acid sequence and structure of SeM and compare them to those of SzPSe and other streptococcal M and M-like proteins, to evaluate the opsonic activity and specificity of antibodies to SeM and SzPSe, and finally to determine whether these proteins from different isolates of *S. equi* vary in size as previously observed for the SzP protein family of *S. zooepidemicus*. We report for the first time the amino acid sequence and structure of SeM, the major protective antigen of *S. equi*, and show that it is only distantly related to the SzP family of proteins.

MATERIALS AND METHODS

Cloning, sequencing, and expression of SeM. Bacteria, plasmids, and bacteriophages used in this study are listed in Table 1. Chromosomal DNA of *S. equi* CF32 was partially digested with *Tsp*5091 (New England Biolabs Inc., Beverly, Mass.), and 3- to 8-kb fragments ligated to lambda ZAPII were digested with *Eco*RI (Stratagene, La Jolla, Calif.). After packaging (with the Gigapack II kit; Stratagene) and transfection into *Escherichia coli* XLI-Blue MRF′ (Stratagene), the library was plated, amplified, and stored at −70°C in 7% dimethyl sulfoxide. The library was screened on duplicate nitrocellulose discs by using rabbit 216 antiserum (1:4,000 dilution) to the acid-extracted 41-kDa fragment of SeM. Several reactive plaques were screened until all plaques gave a positive signal. Proteins in these phage lysates were separated by sodium dodecyl sulfate-poly-

* Corresponding author. Mailing address: Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099. Phone: (606) 257-4172. Fax: (606) 257-5169.

TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Bacterium, bacteriophage, or plasmid	Description	Source
S. equi (*S. equi* subsp. *equi*)		
Lexington	Nonencapsulated	Kentucky, 1990
Boldmani		Ireland, 1983
Ir17		Ireland, 1984
G4		Germany, 1986
465351		New York, 1989
709-27	Nonencapsulated mutant of CF32	New York, 1981
F43	U.S. vaccine strain	United States, 1981
CF32	Prototype strain	New York, 1981
e33		New York, 1976
e80		New York, 1980
XI 331		Sweden, 1985
Relic Spirit		Ireland, 1983
S. zooepidemicus (*S. equi* subsp. *zooepidemicus*) W60		New York, 1976
E. coli		
XL1-Blue MRF′		Stratagene
SOLR		Stratagene
NovaBlue		Novagen
BL21(DE3)		Novagen
SEM01	SOLR(pSEM01)	Transformant
SEM02	BL21(DE3)(pSEM02)	Transformant
SzPSe01	BL21(DE3)(pSzPSe01)	Transformant
Bacteriophages		
Lambda ZAPII		Stratagene
Exassist		Stratagene
λ8-10	Lambda ZAPII with SEM	This work
Plasmids		
pSK(−)		Stratagene
pET15b	His tag vector; Ap^r	Novagen
pSEM01	pSK(SeM); excised from λ8-10	This work
pSEM02	pET15b (*Bam*HI PCR fragment of *seM* from pSEM01)	This work
pSzPSe	pET15b (PCR fragment encoding SzPSe)	This work

acrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with serum 216. A plasmid containing a 3.5-kb fragment encoding SeM was excised from a positive phage, and the resulting plasmid was designated pSeM01. Nucleotide sequencing was performed on *Hin*dIII, *Pvu*II, and *Hin*dIII-*Pvu*II fragments of the *S. equi* insert in pSK by automated cycle sequencing. Sequences were aligned and connected by using DNASIS software (Hitachi Software Engineering America, Ltd., San Diego, Calif.).

SeM without its signal sequence was subcloned into the *Bam*HI site of the pET15b (Novagen, Madison, Wis.) expression vector by PCR with pSeM01 as the template and primers SeM-F (gcggatcCGAACTCTGAGGTTAGTCGT) and SeM-R (gcggatccATAGCTTAGTTTTCTTTGCG). The resulting plasmid was designated pSeM02 and transformed into *E. coli* BL21(DE3). Recombinant SeM was isolated from a lysate of BL21 by affinity chromatography on His-Bind Resin (Novagen).

Amino acid sequence determination. Acid-extracted SeM (200 μg) purified as described below was loaded onto a 2-cm-wide slot on a 1.5-mm-thick acrylamide (9%) gel for preparative SDS-PAGE. The gel was run with 0.1 mM thioglycolic acid for 40 min at 100 V before the protein was loaded. Following electrophoresis and electrophoretic transfer to an Immobilon-P membrane (Millipore), the major 41- and 46-kDa fragments of SeM were identified by staining for 1 min with 0.025% Coomassie blue in 40% methanol and 5% glacial acetic acid followed by destaining for 2 min in 30% methanol and 5% glacial acetic acid. The 41-kDa band was excised, and microsequence analysis was performed on a model 477A pulse liquid-phase sequencer (Applied Bio Systems) at the University of Kentucky Macromolecular Synthesis Laboratory.

Sequence and expression of SzPSe. SzPSe was obtained from a DNA fragment of *S. equi* CF32 amplified by PCR with primers based on the 5′ and 3′ sequences of SzPW60 of *S. zooepidemicus* W60 (5′ ACAAAAGGGGAATAAAATGGC 3′) and NCR3 (5′ TTTACCACTGGGGTATAAGGC 3′) and sequenced as described previously (25, 27). SzPSe without its signal sequence was amplified by PCR with primers Se-F (gcggatcCACGTGTATCAGCAGATTCT) and SeM-R and inserted into the *Bam*HI site of pET15b (Novagen). *E. coli* BL21(DE3) was transformed by this plasmid (pSzPSe01), and recombinant SzPSe protein was isolated as described above for SeM.

Protein extraction. M-like protein was extracted from an overnight culture (18 h) of *S. equi* by hot-acid extraction (14) and adsorbed to a column of hydroxyapatite in 10 mM phosphate buffer, pH 7.2. The M-like proteins were eluted in 0.2 M Na_2HPO_4, desalted on Sephadex G-25, and lyophilized. The pellet was dissolved in 25% acetonitrile–0.5% trifluoroacetic acid and loaded on a phenyl reverse-phase column (Bio-Rad, San Francisco, Calif.) connected to a protein purification system (model 650; Waters, Marlborough, Mass.). Protein was eluted by using a linear gradient of 25 to 65% acetonitrile–0.5% trifluoroacetic acid. A dot immunoblot on cellulose nitrate with SeM-specific rabbit antiserum was used to screen peaks. The peak containing SeM protein eluted at a concentration of approximately 42% acetonitrile. Positive peaks from several runs were pooled and further purified on the same column. Purified protein was lyophilized, resuspended in phosphate-buffered saline (PBS), and stored in aliquots at −20°C. Mutanolysin extracts of *S. equi* strains were obtained as described previously (7).

Antisera. Antiserum was raised against SeM purified by a combination of hydroxyapatite and reverse-phase chromatography. A New Zealand White rabbit (216) was injected subcutaneously with 50 μg of SeM in complete Freund's adjuvant, which was followed at 3-week intervals by two similar doses emulsified in incomplete Freund's adjuvant. Serum was harvested at 8 weeks. Rabbit 963 was similarly hyperimmunized with recombinant SeM from *E. coli* sonicate.

Rabbit antisera 3352 and 3353 against recSzPSe were raised in the same way, by using 50-μg doses of a lysate of *E. coli* NovaBlue containing SzPSe. Rabbit Ec was immunized with a lysate of *E. coli* NovaBlue containing plasmid pT7 Blue without an insert.

Adult ICR (Institute of Cancer Research) mice were immunized with 25 μg of SeM purified from *E. coli* sonicate by His tag chromatography. Purified SeM (25 μg) was mixed with 5 μg of mycolic dipeptide and alhydrogel (30%) and administered subcutaneously in a 100-μl dose. Two subcutaneous booster doses containing 25 μg of SeM but no mycolic dipeptide were administered 10 and 20 days later. Mice were bled at 28 days.

All antisera were stored at −70°C until used.

Immunoblotting. Proteins in streptococcal extracts or purified from *E. coli* sonicate were separated by SDS–10% PAGE, electroblotted onto a sheet of nitrocellulose, and incubated in the appropriate antiserum diluted 1:200 in PBS and then in peroxidase-conjugated protein G (1:4,000). Reactive bands were visualized by using 4-chloro-1-naphthol (0.5 mg/ml) as substrate.

ELISA. An acid-extracted fragment (41 kDa) of SeM, purified by preparative electrophoresis on agarose, was used to coat wells (2.5 μg/well) of polystyrene enzyme-linked immunosorbent assay (ELISA) plates (catalog no. 25880; Costar, Cambridge, Mass.). After the wells were washed and blocked in 0.1 M PBS containing 0.05% Tween 20 and 1% bovine serum albumin, mouse or rabbit sera diluted 1:80 and 1:200, respectively, in PBS were added in triplicate to the wells (100 μl/well). After incubation for 3 h at 37°C, bound immunoglobulin G was detected with either peroxidase-conjugated protein G (1:4,000) or rabbit anti-mouse immunoglobulin G followed by *o*-phenylenediamine (0.0001 mM) solution. Mean optical density (OD) values of triplicate readings were corrected by subtracting OD values for wells containing antigen and PBS.

Opsonic assay. Equine neutrophils were separated from freshly collected heparinized horse blood with a discontinuous Percoll gradient (18). Neutrophils from 7 ml of blood were suspended in RPMI medium (Gibco, Grand Island, N.Y.), and 80-μl aliquots (6×10^5 cells) were added in triplicate to wells of a 24-well cell culture cluster (Costar). Each well contained a circular, glass coverslip (12 mm diameter). The cluster was incubated for 2 h at 37°C in 5% CO_2, and the wells were washed once with PBS to remove nonadherent neutrophils.

The test organisms (*S. equi* CF32 and *S. zooepidemicus* W60) were grown overnight at 37°C in Todd-Hewitt broth with 0.2% yeast extract to an OD of 0.6 at 600 nm. Twenty microliters of culture was added to 25 μl of serum, and 450 μl of RPMI was added. After the plate was gently shaken for 30 min at 37°C, the coverslips were washed once with PBS (pH 7.2), fixed in 10% formalin, and stained with Giemsa stain. The numbers of neutrophils with associated streptococci per 100 cells were then counted for each serum and expressed as a percentage. All assays were performed in triplicate. The differences in the opsonic activities of immune and control sera were evaluated statistically by a Student *t* test (unpaired observations) based on the means of three experiments.

Fibrinogen binding assay. Equine fibrinogen (0.5 μg/well) was bound to wells of 96-well polystyrene ELISA plates (Costar). After the wells were washed and blocked, recombinant SeM (0.4 μg/well) and SzPSe (0.1 μg/well) were added in triplicate to separate wells and incubated for 2 h at 37°C. After the wells were washed, 1:80 dilutions of rabbit antisera to the 41-kDa fragment of SeM and to

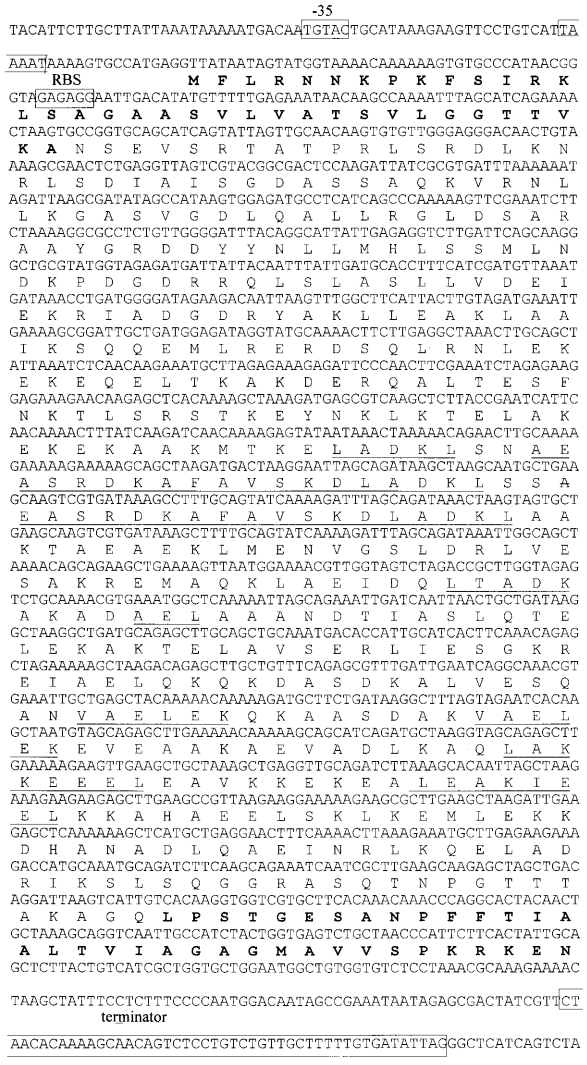

FIG. 1. Nucleotide and deduced amino acid sequences of SeM. Base and amino acid positions are shown on the left. Putative promoter sites and the ribosomal binding site (RBS) are boxed, signal and membrane anchor sequences are shown in boldface type, and repeats are underlined.

recombinant SzPSe were added to the appropriate wells and incubated at 37°C for 2 h. Control wells consisted of wells from which fibrinogen was omitted and wells treated with sera from the same rabbits before immunization. Amounts of specific rabbit antibody that bound to SeM and SzPSe fixed to fibrinogen were detected as described under ELISA above.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences of *SeM* and *SzPSe* are U73162 and U73163, respectively.

RESULTS

Sequence of SeM. Screening of the DNA library of *S. equi* subsp. *equi* in lambda ZAPII with rabbit antiserum 216 against the 41-kDa fragment of SeM revealed several plaques that contained a reactive protein of the expected molecular mass (58 kDa). Nucleotide sequencing was performed on *Hin*dIII, *Pvu*II, and *Hin*dIII-*Pvu*II fragments of a 3.5-kb insert from pSeM01 following subcloning in pSK. Analysis of the connected sequences revealed the presence of one open reading frame of 1,605 nucleotides, forming the *SeM* gene (Fig. 1). Translation of *SeM* revealed a basic (CH +4.5) preprotein of 535 amino acids with a calculated molecular weight of 58,251 and a pI of 8.67. The N-terminal amino acid sequence (residues 37 to 52) was identical to that (NSEVSXTATPRLSRD) obtained by direct microsequencing of the 41-kDa SeM fragment purified from an acid extract of *S. equi.*

The predicted amino acid sequence exhibits features typical of streptococcal surface proteins. The signal sequence is 36 residues. The N terminus of the mature protein has a net positive charge. The anchor membrane-spanning region and charged tail sequences are similar to those of other group A and C streptococcal sequences (Fig. 2). Two direct repeats (21 residues) are located between residues 226 and 267. Other shorter direct repeats varying in length from three to six residues occur in the carboxy-terminal half of the molecule. Analysis of the secondary structure of the translated protein shows an extensive region of alpha-helix extending approximately from residue 120 to 480. The secondary-structure prediction shows turns in the vicinity of residues 120 and 480 to 500.

Sequence of SzPSe. Nucleotide sequence analysis of the DNA fragment of *S. equi*, amplified by PCR with 5′ and 3′ primers from the prototype sequence of SzP from *S. zooepidemicus* W60, revealed one open reading frame of 1,125 bp, encoding a preprotein of 375 amino acids with a molecular weight of 39,664 and a pI of 5.96 (Fig. 3). A signal cleavage site at amino acid 32 or 33 produces a signal peptide with a length similar to that of streptolysin O and protein G and with 88% homology to the prototype SzPW60 sequence (25). Secondary-structure prediction revealed an extended alpha-helical structure with turns near the N terminus and also in the non-alpha-helical hypervariable region.

Reactivities of recombinant SeM and SzPSe in immunoblots. Rabbit 963 antiserum to recSeM reacted with a 58-kDa protein in a mutanolysin extract of *S. equi* and with a slightly larger, 60-kDa protein expressed by *E. coli* BL21 containing pSeM02 (Fig. 4). The same protein bands were recognized by rabbit 216 antiserum to the 41-kDa fragment of SeM. Antiserum to recombinant SzPSe reacted with a 56-kDa protein band in a mutanolysin extract of *S. equi* similar to that expressed by pSzPSe01 in *E. coli* BL21 (Fig. 5). However, a strongly reacting band at about 61 kDa was copurified from the *E. coli* extract. This larger protein may represent a form of SzPSe that contains additional histidine residues. Another explanation is that mutanolysin may not release the entire native protein. The greater-than-predicted molecular mass of SzPSe

SIGNAL SEQUENCES

SeM	MFLRNNKPK FSIRKLSAGAASVLVATSVLGGTTVKA
SzPSe	M AKKEMK FYLRKSAFGLASVSAALLVGAAR VSA
SzPW60	M AKKEMK FYLRKSAFGLASVSAALLVSSA VVAA
ZAG	M EKTKTVSYF LRQSAVGLASVSAAFLV GTSSVG
FA1	MARKNKTKQY SIRKL SVGAA SVLVATGLIGGATVSA
M6	MAKNNTNRHY SLRKLKKGTASVAVALSVIGAGLVVNTNEVSA

MEMBRANE ANCHOR SEQUENCES

SeM	KAGQLPSTGESANPFFTIAALTVIAGAGMAVVSPKRKEN
SzPSe	DTNKLPSTGEATNPFFTAAALAVMAGAGVAAVSTRRKEN
SzPW60	DANKLPSTGEATNPFFTAAALAVMAGAGVAAVSTRRKEN
ZAG	KAKTLPTTGEKANPFFTAAALAIMAGAGALAVTSKRQQD
FA1	DEKKLPSTGETVNPFFTAAGMAGMATAGVVAVGKRKEEN
M6	TKRQLPSTGETANPFFTAAALTVMATAGVAAVVKRKEEN

FIG. 2. Alignment of amino acid sequences in the signal and membrane anchor regions of the SeM and SzPSe proteins of *S. equi*, the M6 protein of *S. pyogenes* (9), the ZAG (11) and SzPW60 (26) proteins of *S. zooepidemicus*, and the FA1 protein of a nonspeciated equine group C streptococcus (22).

```
   1  M  A  K  K  E  M  K  F  Y  L  R  K  S  A  F  G  L  A  S  V
   1  ATGGCAAAAAAAGAAATGAAGTTTTACCTTCGTAAATCAGCCTTTGGGCTAGCTTCAGTA
  21  S  A  A  L  L  V  G  A  A  R  V  S  A  D  S  V  E  S  A  G
  61  TCAGCAGCCCTCTTGGTTGGTGCGGCACGTGTATCAGCAGATTCTGTTGAGTCAGCTGGG
  41  P  V  A  V  A  V  T  D  S  L  D  S  E  A  A  A  T  K  A  E
 121  CCTGTAGCAGTTGCTGTTACAGATAGTTTAGATAGTGAAGCCGCAGCTACAAAGGCAGAA
  61  A  D  L  V  A  A  K  A  D  L  A  A  A  E  V  A  I  T  A  A
 181  GCTGATCTAGTTGCTGCAAAAGCAGACCTAGCAGCAGCAGAAGTAGCAATCACAGCTGCA
  81  K  A  E  F  D  T  A  Q  A  D  L  A  T  A  E  A  T  I  A  E
 241  AAAGCTGAATTTGACACCGCTCAAGCAGACCTAGCTACCGCTGAAGCTACTATCGCAGAG
 101  L  E  Q  K  I  P  E  L  E  K  K  I  Q  E  A  Q  E  K  L  N
 301  CTTGAGCAAAAAATTCCTGAGTTAGAGAAAAAAATTCAAGAAGCTCAGGAAAAACTAAAC
 121  Y  E  N  R  P  S  P  K  R  V  G  S  D  D  E  D  D  T  V  A
 361  TATGAAAATCGACCATCACCTAAACGTGTGGGCAGTGATGATGAAGATGACACAGTAGCT
 141  R  K  L  M  S  E  K  E  A  L  K  A  E  L  Q  K  T  K  E  A
 421  AGAAAGCTTATGTCAGAAAAGGAAGCTCTTAAGGCTGAGCTTCAAAAGACTAAAGAAGCG
 161  L  D  T  A  K  R  A  Y  A  G  I  E  E  R  K  Q  V  A  A  T
 481  CTAGATACAGCTAAAAGAGCTTATGCTGGCATCGAAGAAAGAAAACAAGTAGCAGCAACT
 181  K  L  D  A  A  N  K  A  F  A  G  V  E  E  K  H  A  Q  A  M
 541  AAGCTAGATGCAGCTAACAAGGCTTTTGCTGGAGTTGAAGAAAAGCATGCTCAAGCAATG
 201  A  A  F  G  A  A  F  A  A  Y  K  G  A  V  K  A  E  L  K  A
 601  GCTGCATTTGGAGCAGCATTCGCAGCATACAAGGGAGCTGTTAAGGCTGAATTGAAGGCA
 221  A  G  A  S  D  F  Y  T  K  K  I  D  S  A  D  T  V  D  G  V
 661  GCAGGTGCTAGCGACTTCTACACCAAGAAGATTGACTCTGCCGACACAGTTGACGGTGTT
 241  K  T  L  R  E  M  I  L  D  S  I  A  K  P  E  V  E  P  E  A
 721  AAGACACTCAGAGAAATGATCTTAGACTCAATTGCTAAGCCAGAGGTTGAACCAGAGGCT
 261  K  P  E  P  K  L  E  P  K  P  E  P  K  P  E  P  K  P  E  P
 781  AAGCCTGAACCTAAGCTAGAACCAAAACCTGAGCCAAAACCTGAGCCAAAACCAGAACCT
 281  K  P  E  P  K  P  E  P  K  P  E  P  K  P  E  P  K  P  E  P
 841  AAGCCAGAACCAAAACCTGAGCCTAAGCCAGAGCCAAAACCAGAACCTAAGCCTGAGCCT
 301  K  P  E  P  K  P  E  P  K  P  K  P  Q  P  K  P  A  P  A  P
 901  AAGCCAGAACCAAAACCAGAACCTAAGCCAAAACCTCAACCTAAACCAGCTCCAGCTCCA
 321  K  P  E  A  K  K  E  E  K  K  A  A  P  K  Q  D  T  N  K  L
 961  AAACCTGAAGCTAAGAAGGAAGAGAAGAAAGCAGCGCCTAAGCAAGACACCAACAAATTG
 341  P  S  T  G  E  A  T  N  P  F  F  T  A  A  A  L  A  V  M  A
1021  CCATCAACAGGTGAAGCTACAAATCCATTCTTCACCGCGGCAGCTCTTGCAGTTATGGCA
 361  G  A  G  V  A  A  V  S  T  R  R  K  E  N
1081  GGTGCAGGCGTGGCTGCAGTGTCAACAAGACGCAAAGAAAACTAA
```

FIG. 3. Nucleotide and deduced amino acid sequences of SzPSe. Signal and membrane anchor sequences are shown in boldface type.

seen upon SDS-PAGE is probably due to the presence of proline-rich repeats, which are known to cause anomalously slow migration during electrophoresis, as previously seen for the prototype SzP protein and in numerous other studies of proteins with proline-containing repeats (25).

Figure 6 shows the immunoblot profiles of mutanolysin extracts of a series of *S. equi* isolates collected at different times in the United States and Europe. Antiserum to recSeM recognized two protein bands of 58 and 56 kDa in all the extracts. The doublet form of SeM, although not evident in Fig. 4, is characteristic of most mutanolysin extracts of *S. equi* (7). Antiserum to SzPSe recognized a band of about 56 kDa in all these extracts. The remarkable uniformity in size of the reactive bands from these isolates of *S. equi* contrasts with the previously observed varied sizes and band patterns of the SzP proteins of *S. zooepidemicus* and is consistent with the proposed clonal character of isolates of *S. equi* (12).

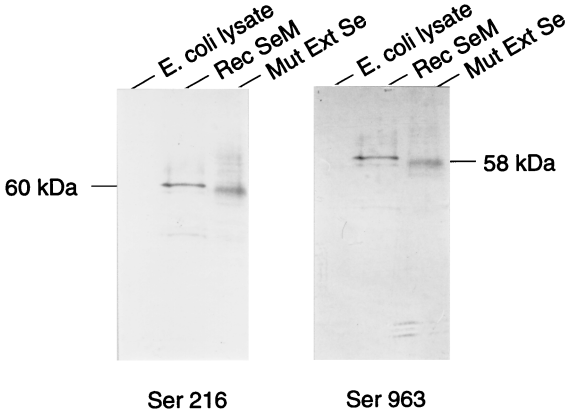

FIG. 4. Immunoblots showing reactions of a lysate of *E. coli* BL21, SeM02 (Rec SeM), and a mutanolysin extract of *S. equi* (Mut Ext Se) with antisera 216 and 963 to SeM and recombinant SeM, respectively.

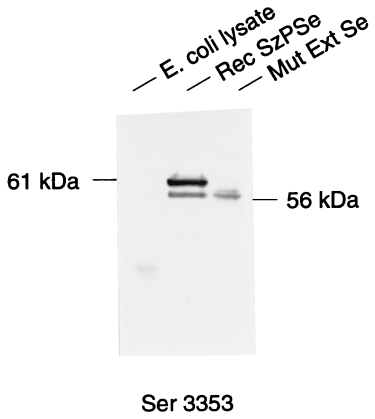

FIG. 5. Immunoblot showing reactions of a lysate of *E. coli* BL21, SzPSe01 (Rec SzPSe), and a mutanolysin extract of *S. equi* (Mut Ext Se), with antiserum 3353 to recombinant SzPSe.

Opsonogenicity of recombinant M-like proteins SeM and SzPSe. Sera from mice immunized with purified recombinant SeM showed ($P < 0.01$) opsonic activity for *S. equi* 15 times greater than did non-immune mouse sera (Table 2). These sera also showed strong antibody responses to the 41-kDa fragment of SeM by ELISA. In contrast, rabbits immunized with SzPSe showed only small increases in opsonic activity for *S. equi*, although they had strong ELISA responses to an acid extract of *S. equi* that contained this protein. Since the opsonic activity for *S. equi* of rabbit Ec immunized with *E. coli* BL21 lysate was similar to those of rabbits 2F and 3353 following immunization, it is probable that the small increases noted were nonspecifically related to hyperimmunization. The lack of opsonization of *S. equi* by SzPSe antisera was not due to an inability to react with SzPSe on the cell surface. The antisera reacted strongly in an ELISA with whole cells of *S. equi* (data not shown). The opsonic activities of sera from rabbits 2F and 3353 were, how-

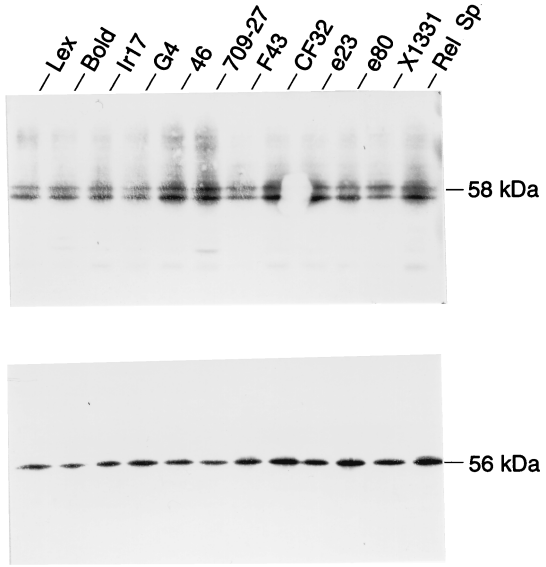

FIG. 6. Immunoblots showing the reactions of mutanolysin extracts of a series of temporally and geographically separated isolates of *S. equi* with antisera to recombinant SeM (upper panel) and to recombinant SzPSe (lower panel). Estimated molecular masses are shown to the right of the figure. Lex, Lexington; Bold, Boldmani; Rel Sp, Relic Spirit.

TABLE 2. Opsonic activities of SeM and SzPSe antisera for *S. equi* and *S. zooepidemicus*

Serum (sera)	Antigen	ELISA OD	% Neutrophils with[c]:	
			S. equi	*S. zooepidemicus*
Mouse				
1, 2, 3	None	0.3[a]	0.7 ± 1.1[c]*	Not done
4, 8, 9, 13	recSeM	2.3[a]	17 ± 4.8*	Not done
Rabbit				
216	Preimmune	0.3[a]	4.1 ± 2.5**	19.0 ± 1.6
216	SeM (41-kDa fragment)	>2.5[a]	20.3 ± 1.2**	20.3 ± 1.2
2F, 3353	Preimmune	0.3[b]	4.3 ± 2.0	25.5 ± 1.5***
2F, 3353	recSzPSe	2.0[b]	7.0 ± 1.5	42.5 ± 2.1***
Ec	Preimmune	0.3[b]	5.6 ± 1.5	22.0 ± 1.4
Ec	*E. coli* lysate	0.3[b]	7.3 ± 1.2	21.0 ± 0.8

[a] The antigen was a purified 41-kDa fragment of SeM (0.02 μg/well).
[b] The antigen was an acid extract of *S. equi* (1.0 μg/well). Mouse and rabbit sera were diluted 1:80 and 1:200, respectively.
[c] Means ± standard deviations from three experiments. Neutrophils were incubated with *S. equi* or *S. zooepidemicus* for 30 min. Only the values marked with asterisks were significantly different. *, $P < 0.01$; **, $P < 0.01$; ***, $P < 0.001$.

ever, significantly ($P < 0.001$) increased for *S. zooepidemicus* following immunization with SzPSe.

Fibrinogen binding. Both SeM and SzPSe showed strong binding to equine fibrinogen immobilized on wells of ELISA plates. ELISA values (means ± standard deviations) for SeM and SzPSe bound to fibrinogen, after correction for nonspecific binding of the proteins to blocked-well surfaces, were 0.9 ± 0.1 and 2.0 ± 0.1, respectively. The corrected values for both were 0.1 ± 0.1 when preimmune sera were used to assay for binding of the streptococcal proteins.

Homologies. With the exception of signal and membrane anchor sequences, no homology of SeM or SzPSe with group A or G M protein sequences in the GenBank database was detected. The homologies of these proteins to the prototype M6 sequence of *Streptococcus pyogenes* and to the FAI, ZAG, and SzPW60 proteins of group C streptococci are shown in Fig. 2. SeM and SzPSe show no homology other than between their signal (39% identity) and membrane anchor (66% identity) sequences. As expected, SzPSe shows high homology (85% identity) to the prototype protein SzPW60 from *S. zooepidemicus* W60. Their sequences differ at the signal sequence cleavage site, at the hypervariable region between residues 115 and 165 (27), and in the number of carboxy-terminal PEPK repeats. Comparison of the signal sequences of SeM and M6 reveals differences in length (36 and 42 residues, respectively) and only 35% identity. However, the SeM signal sequence shows only 34 and 52% identities to the group C signal sequences of ZAG and FAI, respectively, suggesting that the group C genes may be as distantly related to each other as they are to the *emm* gene family of *S. pyogenes* for which there is high homology between M protein signal sequences (8). The membrane anchor sequences vary from only 46% identity between M6 and FAI to almost perfect identity between SeM, SzPSe, and SzPW60.

DISCUSSION

Although the presence of a hot-acid-extractable opsonogenic and protectively immunogenic M-like protein (SeM) on *S. equi* has been recognized for many years, its sequence, structure, and relationship to other M-like proteins were not known until the present study. Functionally, SeM has an antiphagocytic action similar to that of the group A M proteins in that C3b deposition on the bacterial surface is inhibited and fibrinogen is actively bound (4, 10). Antibodies to this protein opsonize *S. equi* but not the closely related *S. zooepidemicus*. Structurally, SeM lacks the prominent A and B repeat regions characteristic of many of the group A M proteins but otherwise shows a similar secondary structure. Unlike those of the group A M proteins, the N terminus of SeM has a net positive charge. Thus, although satisfying the criterion of type-specific opsonogenicity necessary for the designation M protein, SeM is clearly only a distant relative of the group A M protein family. Interestingly, M proteins more closely related to the group A M protein family have been detected on groups C and G streptococci of human but not animal origin (3, 17, 20, 21), and it has been suggested that this is a result of horizontal transfer of *emm* genes between these streptococcal groups. Clearly, horizontal transfer would be most likely to occur within the same host species, a possibility supported by studies of group A streptococci (2). The very limited homologies observed between the M-like proteins of the highly host-adapted human and animal pyogenic streptococci may be explained by the hypothesis that they have evolved in different hosts with little opportunity for horizontal gene transfer.

SzPSe, the second M-like protein of *S. equi*, is clearly an allele of SzP, the protective M-like protein and typing antigen of *S. zooepidemicus*, and is only distantly related to SeM. Although antisera to SzP and SzPSe are strongly cross-reactive (25), SzP antibodies are not protective against *S. equi* (25, 26). The SzP family as expressed in the Moore and Bryans serovars 1 to 15 shows two forms of N-terminal variants (N1 and N2), at least five variants of a central non-alpha-helical hypervariable region (HV1 to HV5), and a variable number of PEPK repeats in the carboxy terminus (27). SzP has an N2 amino-terminal sequence and an HV4 hypervariable region, suggesting that the clone that now comprises *S. equi* was derived from an ancestral strain of *S. zooepidemicus* that had this SzP variant. This conclusion is consistent with MEE data that have indicated that *S. equi* shares a much closer genetic relationship with a few MEE phenotypes of *S. zooepidemicus*, the putative archetypal species, than it does with the majority of more genetically diverse strains (12).

A protein cross-reactive with SeM has not been detected in extracts of more than 200 equine isolates of *S. zooepidemicus* expressing a great variety of SzP phenotypes. The presence of the SeM protein on *S. equi* with its potent antiphagocytic activity clearly would have a very significant enhancing effect on virulence, and neutralization of this effect by opsonic antibody to SeM would explain the specificity of the humoral protection characteristic of *S. equi* infection in both mice and horses (1, 25). Also, assuming a dominating antiphagocytic role for SeM makes it possible to explain how antiserum to SzPSe is not opsonic for *S. equi* (Table 2) yet is effective in opsonizing *S. zooepidemicus*, which lacks a homolog of SeM.

The clonality of temporally and geographically separated isolates of *S. equi* is unambiguously demonstrated in Fig. 6. SeM and SzPSe proteins from all isolates are the same size on immunoblots with antisera raised against the recombinant proteins. The remarkable uniformity in molecular mass of the SzPSe protein contrasts strongly with the varied blot pattern produced by SzP proteins from different isolates of *S. zooepidemicus* (23). The retarding effect of the proline-rich domains of SzPSe on its migration during SDS-PAGE is also clearly evident in Fig. 5 and 6. Although smaller than SeM by 18 kDa, SzPSe bands at almost the same position on the gel.

The contribution of SzPSe to the virulence of *S. equi* is not

yet known. Binding of fibrinogen would be expected to reduce phagocytosis. Pretreatment of *S. equi* with SeM-specific antibody reduces fibrinogen binding by about 70%, suggesting that SeM is more important for fibrinogen binding than SzPSe (4). Nevertheless, a piece of evidence for substantial binding by SzPSe is the lack of a proportionate reduction in fibrinogen binding by *S. equi* 19, a strain that expresses normal amounts of SzPSe but only about 4% of the normal amount of SeM yet binds 36% of the amount of fibrinogen bound by *S. equi* CF32 (4, 5). The lack of sequence homology between the domains of SeM and SzPSe external to the cell wall suggests that fibrinogen binding to these alpha-helical proteins and to others such as the group C FAI protein and members of the group A M protein family is mediated by molecular features not directly related to simple linear amino acid sequence. This is not surprising given that functions such as the antiphagocytic property of the group A M proteins are not dependent on conservation of sequence (19).

The FAI protein is of particular interest with respect to the M-like proteins of *S. equi* and *S. zooepidemicus*, because not only is it produced on a group C streptococcus isolated from a horse but it has an extensive C repeat region with 40 to 50% sequence identity to the B repeats of M49 and the C repeats of the M12 proteins of group A streptococci. No homology to the C repeat of FAI is evident in SeM or SzPSe. It has been suggested that the homology of the FAI protein to the M49 and M12 repeats has arisen from horizontal interstreptococcal species transfer (22). Since *S. pyogenes* is rarely isolated from nonprimates, a more plausible explanation is that the FAI-positive group C streptococcus was possibly of human origin and accidentally present in the horse at the time it was isolated.

The availability of the amino acid sequence of SeM now makes possible new studies on its role in virulence and pathogenesis and on the epitopes and domains of the protein relevant to opsonic and mucosal protective responses. The availability of the DNA sequence in combination with PCR will also be of great value in characterizing the precursor of the *SeM* gene in *S. zooepidemicus* and perhaps provide some understanding of the genetic process that produced the more virulent clonal derivative now known as *S. equi.*

ACKNOWLEDGMENTS

The technical assistance of Juan Wang is gratefully acknowledged.

This research was supported by USDA-NRIGP grant 9501837 and by the Keeneland Association.

REFERENCES

1. **Bazely, P. C.** 1942. Studies with streptococci. 4. Cross immunity to *Streptococcus equi*. Aust. Vet. J. **18:**189–194.
2. **Bessen, D. E., and S. K. Hollingshead.** 1994. Allelic polymorphism of emm loci provides evidence for horizontal gene spread in group A streptococci. Proc. Natl. Acad. Sci. USA **91:**3280–3284.
3. **Bisno, A. L., C. M. Collins, and J. C. Turner.** 1997. M-proteins of group C streptococci isolated from patients with acute pharyngitis, p. 745–748. *In* T. Horaud, M. Sicard, A. Bouvet, R. Leclerq, and H. DeMonclos (ed.), Streptococci and the host. Plenum Press, New York, N.Y.
4. **Boschwitz, J. S., and J. F. Timoney.** 1994. Characterization of the antiphagocytic activity of equine fibrinogen for *Streptococcus equi* subsp. *equi*. Microb. Pathog. **17:**121–129.
5. **Boschwitz, J. S., and J. F. Timoney.** 1994. Inhibition of C3 deposition on *Streptococcus equi* subsp. *equi* by M protein: a mechanism for survival in equine blood. Infect. Immun. **62:**3515–3520.
6. **Fischetti, V.** 1994. Nomenclature of M and M-like genes, p. 109. *In* A. A. Totolian (ed.), Pathogenic streptococci. Present and future. Lancer Publications, St. Petersburg, Russia.
7. **Galán, J., and J. F. Timoney.** 1988. Immunologic and genetic comparison of *Streptococcus equi* isolates from the United States and Europe. J. Clin. Microbiol. **26:**1142–1146.
8. **Haanes-Fritz, E., W. Kraus, V. Burdett, J. B. Dale, E. H. Beachey, and P. P. Cleary.** 1988. Comparison of the leader sequences of group A streptococcal M protein genes. Nucleic Acids Res. **16:**4667–4677.
9. **Hollingshead, S. K., V. A. Fischetti, and J. R. Scott.** 1986. Complete nucleotide sequence of type 6 M protein of the group A streptococcus. J. Biol. Chem. **261:**1677–1686.
10. **Jacks-Weis, J., Y. Kim, and P. P. Cleary.** 1982. Restricted deposition of C3 on M4 group A streptococci: correlation with resistance to phagocytosis. J. Immunol. **128:**1897–1902.
11. **Jonsson, H., H. Lindmark, and B. Guss.** 1995. A protein G-related cell surface protein in *Streptococcus zooepidemicus*. Infect. Immun. **63:**2968–2975.
12. **Jorm, L. R., D. N. Love, G. D. Bailey, G. M. McKay, and D. A. Briscoe.** 1994. Genetic structure of populations of beta-haemolytic Lancefield group C streptococci from horses and their association with disease. Res. Vet. Sci. **57:**292–299.
13. **Kilpper-Bälz, R., and K. H. Schleifer.** 1984. Nucleic and hybridization and cell wall composition studies of pyogenic streptococci. FEMS Microbiol. Lett. **24:**355–364.
14. **Lancefield, R. C., and G. E. Perlmann.** 1952. Preparation and properties of type-specific M antigen from group A type 1 haemolytic streptococcus. J. Exp. Med. **96:**71–82.
15. **Moore, B. O., and J. T. Bryans.** 1970. Type specific antigenicity of group C streptococci from disease of the horse, p. 231–238. *In* J. T. Bryans and H. Gerber (ed.), Equine Infectious Diseases II. S. Karger, Basel, Switzerland.
16. **O'Toole, P., L. Stenberg, M. Rissler, and G. Lindahl.** 1992. Two major classes in the M protein family in group A streptococci. Proc. Natl. Acad. Sci. USA **89:**8661–8665.
17. **Podbielski, A., B. Melzer, and R. Lütticken.** 1991. Application of the polymerase chain reaction to study the M protein(-like) gene family in beta-hemolytic streptococci. Med. Microbiol. Immunol. **180:**213–227.
18. **Pycock, J. F., W. E. Allen, and T. H. Morris.** 1987. A rapid procedure for isolation of equine neutrophils. Res. Vet. Sci. **42:**411–412.
19. **Scott, J. R.** 1990. The M protein of group A streptococcus: evolution and regulation, p. 177–203. *In* B. J. Iglewski and V. L. Clark (ed.), The bacteria, vol. XI. Academic Press, New York, N.Y.
20. **Simpson, W. J., J. M. Musser, and P. P. Cleary.** 1992. Evidence consistent with horizontal transfer of the gene (*emm12*) encoding serotype M12 protein between group A and group G pathogenic streptococci. Infect. Immun. **60:**1890–1893.
21. **Simpson, W. J., J. C. Robbins, and P. P. Cleary.** 1987. Evidence for group A-related M protein genes in human but not animal-associated group G streptococcal pathogens. Microb. Pathog. **3:**339–350.
22. **Talay, S. R., M. P. Grammel, and G. S. Chhatwal.** 1996. Structure of a group C streptococcal protein that binds to fibrinogen, albumin and immunoglobulin G via overlapping modules. Biochem. J. **315**(2)**:**577–582.
23. **Timoney, J. F., and M. Mukhtar.** 1992. Variability in the M proteins of equine strains of *Streptococcus equi* subsp. *zooepidemicus*, p. 15–20. *In* W. Plowright, P. D. Rossdale, and J. F. Wade (ed.), Equine Infectious Diseases VI, Cambridge 1991. R & W Publications, Newmarket, England.
24. **Timoney, J. F., M. Mukhtar, J. Galán, and J. Ding.** 1991. M proteins of the equine group C streptococci, p. 160–164. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
25. **Timoney, J. F., A. Umbach, J. E. Boschwitz, and J. A. Walker.** 1995. *Streptococcus equi* subsp. *equi* expresses 2 M-like proteins including a homologue of the variable M-like protective protein of *Streptococcus equi* subsp. *zooepidemicus*, p. 189–193. *In* T. Kumanomido and W. Plowright (ed.), Equine infectious diseases VII. R & W Publications, Newmarket, England.
26. **Timoney, J. F., J. Walker, M. Zhou, and J. Ding.** 1995. Cloning and sequence analysis of a protective M-like protein gene from *Streptococcus equi* subsp. *zooepidemicus*. Infect. Immun. **63:**1440–1445.
27. **Walker, J. A., and J. F. Timoney.** 1995. Application of the polymerase chain reaction to the study of the molecular basis of M-like protein variation in *Streptococcus equi* subsp. *zooepidemicus*, p. 195–200. *In* T. Kumanomido and W. Plowright (ed.), Equine infectious diseases VII. R & W Publications, Newmarket, England.

Editor: V. A. Fischetti