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Identification of Lipoprotein Homologues of Pneumococcal PsaA in the Equine Pathogens *Streptococcus equi* and *Streptococcus zooepidemicus*

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***Streptococcus equi* and *Streptococcus zooepidemicus* are major etiological agents of upper and lower airway disease in horses. Despite the considerable animal suffering and economic burden associated with these diseases, the factors that contribute to the virulence of these equine pathogens have not been extensively investigated. Here we demonstrate the presence of a homologue of the *Streptococcus pneumoniae* PsaA protein in both of these equine pathogens. Inhibition of signal peptide processing by the antibiotic globomycin confirmed the lipoprotein nature of the mature proteins, and surface exposure was confirmed by their release from intact cells by mild trypsinolysis.**

Streptococcus equi subsp. *equi*, the etiological agent of strangles, has been estimated to be responsible for nearly 30% of all reported equine infections worldwide (6). Strangles is characterized by pharyngeal constriction in the horse's upper respiratory tract as a consequence of lymph node swelling and is often accompanied by abscessation. The very closely related organism *Streptococcus zooepidemicus* (*S. equi* subsp. *zooepidemicus*) is a significant cause of equine lower airway disease, foal pneumonia, endometritis, and abortion (6). Despite the considerable animal suffering and economic burden associated with these diseases, there is little information regarding the molecular basis of virulence of these two streptococci, and there are presently no effective vaccines against either organism (6). Most studies have focused on the M-like proteins of these streptococci (36, 37), and that of *S. equi* has been shown to be a fibrinogen-binding protein (31). Recently, other studies have focused on a streptolysin S-like toxin (17), a fibronectin-binding protein (29), and a hyaluronate-associated protein conferring partial protection in murine models of *S. equi* and *S. zooepidemicus* infection (9). The pyogenic streptococci are highly host adapted, so that pathogenicity is likely to depend on many biochemical, immunological, and cellular interactions. Interference with a critical combination of these may be important in the development of protective immunity (6). The characterization of bacterial cell surface proteins vital for host-pathogen interactions is an essential step toward identifying components which are likely to elicit protective immune responses.

Studies have identified a class of at least eight highly homologous (ca. 70% or greater amino acid identity) 35- to 37-kDa proteins in streptococci, including the PsaA protein of *Streptococcus pneumoniae*, the FimA protein of *Streptococcus parasanguis*, and the MtsA protein of *Streptococcus pyogenes* (4, 15, 22, 33). The genes encoding these proteins are located within operons encoding components of putative ATP-binding cassette (ABC) transport systems (13, 16, 22, 24–26, 32, 34). Moreover, these proteins appear to be a subfamily of a larger family of substrate-binding proteins (cluster 9) involved in the transport of metal ions such as iron, manganese, and zinc (2, 3, 10, 13, 14, 18, 22, 25, 28). A characteristic component of the importer ABC systems of gram-positive bacteria is a solute-binding lipoprotein (35), and consistent with this, the streptococcal 35- to 37-kDa proteins are all putative lipoproteins.

A stable nomenclature has yet to be adopted for these streptococcal proteins, so we refer to them herein as metal binding lipoproteins (MBLs). These lipoproteins may be of considerable importance in the physiology and pathogenicity of streptococci, since MBL-deficient mutants of *Streptococcus mutans*, *S. parasanguis*, and *S. pneumoniae* were significantly less virulent than their wild-type parent strains in animal models of disease (4, 5, 24). Consequently, we have investigated the presence of homologous proteins in *S. equi* and *S. zooepidemicus*, because they may also have significance as virulence determinants.

Initially, degenerate PCR primers were designed based upon

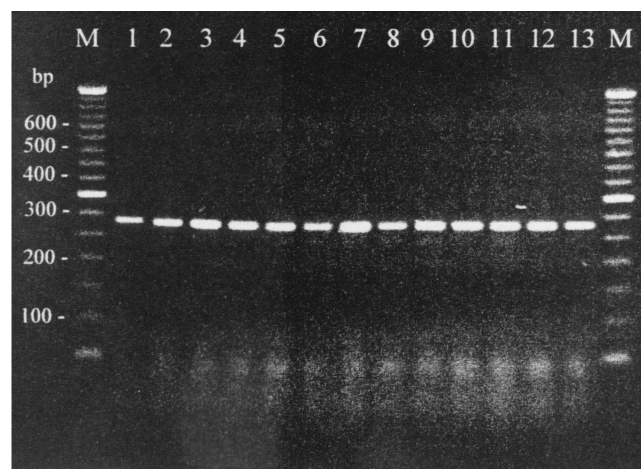

FIG. 1. PCR amplification of fragments of putative MBL genes from *S. equi* and *S. zooepidemicus* and of a *psaA* gene fragment from *S. pneumoniae*. Lanes: M, 50-bp standard ladder (Gibco, Paisley, United Kingdom); 1, *S. pneumoniae* NCTC 11910; 2, *S. equi* NCTC 9682; 3, *S. equi* 1742; 4, *S. equi* 2112; 5, *S. equi* CF32; 6, *S. equi* 4047; 7, *S. equi* 1026; 8, *S. zooepidemicus* NCTC 7023; 9, *S. zooepidemicus* 2809; 10, *S. zooepidemicus* 3682; 11, *S. zooepidemicus* 3685; 12, *S. zooepidemicus* K3; 13, *S. zooepidemicus* 461.

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TABLE 1. Bacterial strains used in this study

Strain[a]	16S–23S RNA gene intergenic spacer type	Host and site of isolation	Geographical location, date of isolation	Reference(s)
S. equi				
NCTC 9682[T]	NT[b]			
CF32	D1	Equine abscess	New York, 1981	37
4047	D1	Equine abscess	Northamptonshire, United Kingdom, 1990	7, 8
1026	D1	Equine nasopharynx	Suffolk, United Kingdom, 1995	7
1742	D1	Equine nasopharynx	Buckinghamshire, United Kingdom, 1995	7
2112	D1	Equine nasopharynx	Kent, United Kingdom, 1997	This study
S. zooepidemicus				
NCTC 7023[T]	NT			
2809	A1	Equine trachea	Berkshire, United Kingdom, 1993	7
3682	A2	Equine trachea	Sussex, United Kingdom, 1992	7
3685	B1	Equine trachea	Sussex, United Kingdom, 1992	7
461	C1	Equine nasopharynx	Suffolk, United Kingdom, 1994	7
K3	D2	Equine lung	Suffolk, United Kingdom, 1993	7
S. pneumoniae NCTC 11910 (serotype 23F)	Not applicable			

[a] NCTC, National Collection of Type Cultures, London, United Kingdom.
[b] NT, not tested.

the highly conserved regions EDPHAW and WEINTE within the published streptococcal MBL sequences corresponding to amino acids 136 to 141 and 223 to 228, respectively, in the pneumococcal PsaA protein (4, 33). PCR with these primers amplified DNA fragments from *S. equi* NCTC 9682 and *S. zooepidemicus* NCTC 7023 that comigrated with a *psaA* fragment amplified with the same primers from *S. pneumoniae* DNA (Fig. 1). Furthermore, amplimers of the same size were also obtained from five disparate clinical isolates of *S. equi* and five disparate isolates of *S. zooepidemicus* (Fig. 1). The *S. zooepidemicus* isolates were selected on the basis of differences in the polymorphisms of their 16S to 23S RNA gene intergenic spacers (Table 1). Since *S. equi* has just one intergenic spacer type (7), strains were selected on the basis of temporal and geographical differences in isolation (Table 1).

Sequencing of the amplified fragments from *S. equi* NCTC 9682 and from *S. zooepidemicus* NCTC 7023 afforded 243 nucleotides of DNA sequence for each organism. The sequences were 97% identical at the nucleotide level, with 100% homology at the translated amino acid level. Homologues of the 81 amino acids derived from these nucleotide sequences were identified by a BLAST search (1) using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST). The translated sequence showed significant homology to internal sequences of all proteins in the MBL family, with greatest homology to MtsA from *S. pyogenes* (22). The DNA sequence of the *S. equi* PCR product was also 100% identical to a contig sequence within the unfinished *S. equi* strain 4047 genome (http://www.sanger.ac.uk/Projects/S_equi/). The contig within which this sequence was located

TABLE 2. Homology of the mature *S. equi* MBL sequence with other streptococcal MBLs and members of the cluster 9 solute binding protein family[a]

Organism and protein	% Identity	% Similarity	Length (amino acids)[b]	BLAST E value	Reference or accession no.[c]
Streptococcus pyogenes MtsA	92	95	290	1×10^{-153}	22
Streptococcus sanguis SsaB	78	88	290	1×10^{-133}	19
Streptococcus oralis PsaA	77	88	290	1×10^{-132}	AF248237
Streptococcus anginosus PsaA	77	88	290	1×10^{-132}	AF248235
Streptococcus pneumoniae PsaA	76	87	290	1×10^{-131}	4
Streptococcus sp. “PsaA”[d]	76	88	290	1×10^{-131}	34
Streptococcus crista ScbA	77	88	289	1×10^{-130}	11
Streptococcus mitis PsaA	76	88	290	1×10^{-130}	AF248236
Streptococcus parasanguis FimA	75	88	290	1×10^{-130}	15
Streptococcus gordonii ScaA	76	88	289	1×10^{-130}	26
Streptococcus mutans LraI	72	84	285	1×10^{-119}	24
Enterococcus faecalis EfaA	58	75	285	1×10^{-96}	30
Staphylococcus epidermidis SitC	51	69	288	2×10^{-79}	10
Yersinia pestis YfeA	35	56	285	1×10^{-48}	3
Synechocystis sp. MntC	32	57	277	2×10^{-45}	2
Bacillus subtilis YcdH	30	48	303	4×10^{-30}	18, 27
Streptococcus pneumoniae AdcA	28	48	294	1×10^{-27}	14

[a] Homologies were determined by using BLAST (1).
[b] Number of amino acids over which homology with the 290-amino-acid *S. equi* sequence was calculated.
[c] GenBank accession numbers obtained from I. Jade and J. Casal.
[d] This sequence was originally attributed to *S. pneumoniae* (33, 34).

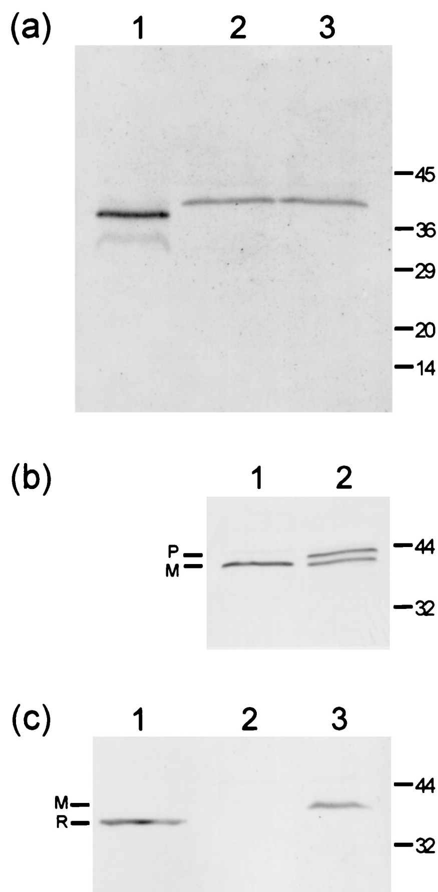

FIG. 2. Western blot analyses demonstrating *S. equi* and *S. zooepidemicus* MBL expression (a), lipoprotein modification (b), and localization (c) using an anti-PsaA antibody. Cell extracts or supernatants were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with polyclonal anti-PsaA antibody. The positions of molecular mass markers are shown (in kilodaltons) at the right-hand side of each panel. (a) Lanes: 1, *S. pneumoniae*-positive control; 2, *S. equi*; 3, *S. zooepidemicus*. (b) Lanes: 1, *S. zooepidemicus* control growth; 2, *S. zooepidemicus* grown in the presence of globomycin. P, prolipoprotein; M, mature lipoprotein. (c) Lanes: 1, supernatant from cells of *S. zooepidemicus* treated with trypsin; 2, supernatant from *S. zooepidemicus* cells incubated without trypsin; 3, SDS extract of untreated *S. zooepidemicus* cells. R, released protein; M, mature lipoprotein.

contained a putative open reading frame (ORF) encoding a protein of 310 amino acids with 89% identity to MtsA of *S. pyogenes*. To verify the presence of the *mbl* gene in *S. equi*, contig sequences corresponding to the MBL signal peptide region and to the 5′ end of the adjacent, downstream ORF were used to design PCR primers which allowed the amplification and sequencing of a DNA fragment encoding the entire mature MBL. The gene contains a "lipobox" motif (MLVAC ↓ S) conforming to that directing lipoprotein cysteine modification in gram-positive bacteria (35). The 290 amino acids deduced for the mature protein sequence (starting from the N-terminal lipobox cysteine) were used in a BLAST search of the available databases. Highly significant homologies (72 to 92% identity) were found with the streptococcal MBLs, most notably with MtsA of *S. pyogenes* (22), and lower homologies (28 to 58% identity) were found with the more distant relatives within the cluster 9 binding proteins (Table 2). Secondary structure analysis using PSIPRED (23; http://insulin.brunel.ac.uk/psipred/) predicted that the major helix or strand features of PsaA (28) are also conserved in the *S. equi* MBL.

BLAST searches of the *S. equi* genome database with the sequences of the *S. pyogenes* MtsB and MtsC proteins revealed homologues of each (80% identity over 197 amino acids for MtsB; 89% identity over 275 amino acids for MtsC), with the MtsB sequence located downstream of *mbl* on the same contig. The nucleotide sequence in the region between the *mbl* gene and the *mtsB* homologue in *S. equi* was also highly homologous to that found between *mtsA* and *mtsB* of *S. pyogenes* and which was previously identified as a putative stem-loop transcription terminator for the *mtsA* gene (22). This therefore suggests that *mbl*, like *mtsA*, is transcribed both individually and as part of a polycistronic message.

Expression of the MBL homologues in *S. equi* and *S. zooepidemicus* was analyzed by Western blotting as previously described (20). Rabbit polyclonal anti-PsaA antiserum (12) cross-reacted with a polypeptide of ca. 38 kDa in extracts of both *S. equi* and *S. zooepidemicus* (Fig. 2a, lanes 2 and 3), whereas a strong reaction with PsaA was detected at ca. 36 kDa in the *S. pneumoniae* control lane (Fig. 2a, lane 1). Approximately twice as much *S. equi* cell extract was needed to produce band intensities comparable to that of *S. zooepidemicus*. Because the MBL sequences from *S. equi* and *S. zooepidemicus* are nearly identical, the weaker reaction in the *S. equi* extract may be due to lower expression of this protein under the growth conditions employed. Growth of *S. equi* in different culture media did not result in increased recovery of the MBL (data not shown). The anti-PsaA antiserum cross-reacted with polypeptides of ca. 38 kDa in extracts of all of the strains of *S. equi* and *S. zooepidemicus* listed in Table 1 (data not shown). Antiserum raised against ScaA, the *Streptococcus gordonii* MBL (26), also cross-reacted with *S. equi* and *S. zooepidemicus* polypeptides with the same molecular masses as those detected with the anti-PsaA antiserum (data not shown). Cumulatively, these results strongly indicate the expression of MBL homologues in *S. equi* and *S. zooepidemicus*.

To confirm the predicted lipoprotein modification of their MBLs, *S. equi* and *S. zooepidemicus* were grown in the presence of the antibiotic globomycin, which specifically inhibits cleavage of lipoprotein signal peptides by signal peptidase II (21). As expected, globomycin treatment of *S. zooepidemicus* resulted in the appearance of an additional, slightly larger polypeptide (Fig. 2b) that cross-reacted with the polyclonal anti-PsaA antiserum and is attributed to the accumulation of the prolipoprotein form of the MBL. Comparable results were obtained for *S. equi* (data not shown). To confirm the surface accessibility of the MBL, whole bacterial cells were subjected to mild digestion with trypsin as previously described (20). As shown in Fig. 2c, truncated forms of the anti-PsaA cross-reactive protein were released from cells of *S. zooepidemicus* in the presence, but not the absence, of trypsin. The size of the released product is consistent with cleavage of the protein at one of the several potential trypsin cleavage sites close to the N terminus of the mature MBL. Similar experiments have shown the release by papain of a truncated form of MtsA from *S. pyogenes* (22).

The results presented here thus provide genetic and biochemical evidence that a selection of disparate isolates of *S. equi* and *S. zooepidemicus* all produce proteins homologous to the PsaA protein of *S. pneumoniae* and other related MBLs. The expression and surface accessibility of this lipoprotein were confirmed serologically in both organisms. Because these proteins are thought to be substrate-binding lipoproteins participating in metal transport systems (13, 22) it seems likely that the new members of this family described herein are also encoded by genes located within operons for ABC transport systems, and this is further supported by the identification of

homologues of MtsB and MtsC of *S. pyogenes* within the unfinished *S. equi* genome. These transport systems could play vital roles in the acquisition of nutrients in the equine host. Because these putative virulence factors may represent novel therapeutic targets in *S. equi* and *S. zooepidemicus*, further studies to characterize their function are now in progress.

Nucleotide sequence accession number. The EMBL accession number for the nucleotide sequence described in this work is AJ249889.

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