Short communication: Conservation of *Streptococcus uberis* adhesion molecule and the *sua* gene in strains of *Streptococcus uberis* isolated from geographically diverse areas

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ABSTRACT

The objective was to identify and sequence the *sua* gene (GenBank no. DQ232760; <http://www.ncbi.nlm.nih.gov/genbank/>) and detect *Streptococcus uberis* adhesion molecule (SUAM) expression by Western blot using serum from naturally *S. uberis*-infected cows in strains of *S. uberis* isolated in milk from cows with mastitis from geographically diverse areas of the world. All strains evaluated yielded a 4.4-kb *sua*-containing PCR fragment that was subsequently sequenced. Deduced SUAM AA sequences from those *S. uberis* strains evaluated shared >97% identity. The pepSUAM sequence located at the N terminus of SUAM was >99% identical among strains of *S. uberis*. *Streptococcus uberis* adhesion molecule expression was detected in all strains of *S. uberis* tested. These results suggest that *sua* is ubiquitous among strains of *S. uberis* isolated from diverse geographic locations and that SUAM is immunogenic.

Key words: *Streptococcus uberis*, *Streptococcus uberis* adhesion molecule, *sua* gene sequencing, mastitis, dairy cow

Short Communication

Streptococcus uberis is an important environmental mastitis pathogen accounting for up to 33% of clinical mastitis in the United Kingdom (Hillerton et al., 1993). In the United States, *S. uberis* is isolated frequently from mammary glands of multiparous cows during the nonlactating period and causes a large proportion of streptococcal mastitis in heifers and multiparous cows during the peripartum period (Oliver, 1988; Leigh, 1999; Oliver et al., 2005). *Streptococcus uberis* mastitis has been and continues to be a major contributor to

the estimated \$2 billion annual cost of mastitis in the United States.

To develop an effective vaccine for the control of *S. uberis* mastitis, knowledge of its virulence factors and their influence on pathogenesis is essential. Adherence, internalization, and intracellular persistence of *S. uberis* into bovine mammary epithelial cells (BMEC) are considered important virulence mechanisms for the establishment of IMI in dairy cows (Almeida and Oliver, 2006; Tamilselvam et al., 2006; Patel et al., 2009). *Streptococcus uberis* adhesion molecule (SUAM) is a key molecule in adherence to and internalization of *S. uberis* into BMEC in vitro (Almeida et al., 2006; Patel et al., 2009). *Streptococcus uberis* adhesion molecule has affinity for bovine lactoferrin (LF); a molecular bridge between SUAM-LF and a putative LF receptor on BMEC enhanced adherence and internalization of *S. uberis* into host cells (Patel et al., 2009).

The *sua* gene, encoding for the complete SUAM protein, was cloned, sequenced, and expressed as a recombinant protein in *Escherichia coli* (Prado et al., 2011). Bovine serum antibodies against recombinant SUAM (rSUAM) and pepSUAM, a 17-AA peptide located at the N terminus of SUAM, inhibited adherence to and internalization of *S. uberis* into mammary epithelial cells (Almeida et al., 2006; Prado et al., 2011).

The value of SUAM as a potential vaccine antigen to control *S. uberis* mastitis will depend on its conservation among strains of *S. uberis*. Objectives of the present study were to sequence and compare *sua* genes and their adjacent regions as well as detect the presence of SUAM by Western blot using serum from naturally *Streptococcus uberis*-infected cows in *S. uberis* strains isolated from diverse global locations.

Frozen stocks of strains of *S. uberis* isolated from cows with clinical or subclinical mastitis in the United States, United Kingdom, Australia, and New Zealand were grown in Todd Hewitt broth (THB; Difco Laboratories Inc., Detroit, MI) overnight at 37°C and genomic DNA was isolated using a Wizard Genomic DNA puri-

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Table 1. Primers for PCR and sequencing reactions^{1,2}

Sequence	Purpose	Expected PCR product size (bp)
<i>sua</i> -U aaaatcttAAGACGTAGTGAACGCATGG	PCR and sequencing	4,408
<i>sua</i> -D ccaagcttACTAACCAACGTAAACCTAA	PCR and sequencing	
<i>sua</i> -U2 AGTCTCCAACCAAACAGTCG	Sequencing	
<i>sua</i> -D2 GTTCTTGCAAGTTGTGTCCG	Sequencing	
<i>sua</i> -U3 CTAAAACTTGCTGAAGCAAC	Sequencing	3,569
<i>sua</i> -D3 ACTTTCATAATCTCTTGTGC	Sequencing	
<i>sua</i> -U4 GGTGCTCAAGAGTTTGCTCG	Sequencing	
<i>sua</i> -D4 TGTGGAAGTTGAGCTTTTGC	Sequencing	
<i>sua</i> -5F cggaaTTCATGACAGTTGCTACTAAAGG	PCR	2,971
<i>sua</i> -3R ttaagcTTACACGTCCACCACGGATA	PCR	
<i>sua</i> -1 gggaatTCATTTGGTAGGAGTGGCTGT	PCR	
<i>sua</i> -4 ggaagcTTGGTTGATATAGCACTTGGTG	PCR	
<i>sua</i> -499 ggtcgacggggggggATTAGCACTCTTCAAG	Sequencing	
<i>sua</i> -699 ggtcgacggggggggGCTCCAAAAATCAGCA	Sequencing	

¹Some primers were synthesized with a 5' sequence tag (lowercase letters) to incorporate a particular restriction enzyme site.

²Sequences were obtained from *Streptococcus uberis* O140J strain genome sequence data published at <http://www.sanger.ac.uk/Projects/Uberis/> and *S. uberis* UT888 strain [National Center for Biotechnology Information (NCBI accession number D0232760; <http://www.ncbi.nlm.nih.gov/genbank/>).

fication kit (Promega Corp., Madison, WI). The primer pair *sua*-U and *sua*-D was used for PCR amplification of a 4.4-kb *sua*-containing DNA fragment, and primers used for sequencing are shown in Table 1.

The PCR reactions to amplify the 4.4-kb *sua*-containing DNA fragment were as follows: 50 μ L of Taq DNA polymerase reaction mix (Invitrogen Inc., Carlsbad, CA) with a final concentration of 1 $\times$ High Fidelity PCR buffer, 200 μ M concentrations of each deoxyribonucleotide triphosphate (dNTP), a 1.5 mM concentration of MgCl₂, 0.3 μ M concentrations of each forward and reverse primer, approximately 100 ng of genomic DNA, 1 unit of Platinum Taq DNA polymerase High Fidelity, and water. The PCR reaction conditions were as follows: cycle 1 (1 $\times$): 95.0°C for 3 min; cycle 2 (30 $\times$): 95.0°C for 30 s, 57.0°C for 30 s, and then 68.0°C for 4.5 min; and finally cycle 3 (1 $\times$): 68.0°C for 10 min. The PCR fragments were electrophoresed in a 0.8% E-Gel for DNA (Invitrogen Inc.) using a TrackIt 1 kb DNA ladder (Invitrogen Inc.) as molecular marker.

The *sua*-containing PCR fragments were sequenced using the Big Dye Terminator Sequencing kit v3.1 [Applied Biosystems Inc. (ABI), Foster City, CA]. Sequencing was run on an ABI 3730 sequencer. Sequence analysis and alignment were conducted using DNA Star Lasergene 8 software suite (DNASTAR Inc., Madison, WI). The ORF sequence of *sua* was codon optimized, synthesized, and cloned into the pET47b vector (kanamycin resistant) between restriction enzymes *Nde*I and *Eco*RI of the multiple cloning site (Blue Heron Biotechnology Inc., Bothell, WA). The *sua* insert (2,676 bp) was cut out and cloned into the pFLEX30 vector between *Nde*I and *Pst*I sites. The plasmid (pFLEX/SUAM with kanamycin resistance marker) was enriched and puri-

fied from *E. coli* DH5 α , and transformed into *E. coli* BL21(DE3)/Star for expression of rSUAM. Recombinant SUAM was expressed as a fusion polypeptide (889 AA; 97,774 Da) containing 11 AA of protective peptide fused into the N terminus of mature SUAM. Expression of rSUAM was induced by shifting the growth temperature to 42°C for 3 h.

Bacterial proteins (10 μ g/lane) from 17 strains of *S. uberis* were electrophoresed on 10% polyacrylamide gels in the presence of SDS using Mini-PROTEAN 3 Cell (Bio-Rad Laboratories Inc., Hercules, CA) as previously described (Laemmli, 1970). Gels were either silver (Oakley et al., 1980) or Coomassie Brilliant Blue stained (CBB-G250; Fisher Scientific, Fair Lawn, NJ) as previously described (Neuhoff et al., 1988; Neumann, 1996). A third and fourth gel were transferred onto nitrocellulose membranes (Midwest Scientific Laboratory Equipment & Supplies, Valley Park, MO) as previously described (Towbin et al., 1979) using a Trans-Blot SD Semi-Dry Electrophoresis Transfer Cell (Bio-Rad Laboratories Inc.). Membranes were blocked (5% nonfat dry milk) and probed with rabbit anti-SUAM polyclonal serum (1:1,000) or bovine anti-*S. uberis* polyclonal serum (1:100) at room temperature for 1 h. Blots were washed 3 times with PBS-0.05% Tween-20 (pH 7.2), incubated with peroxidase-labeled goat anti-rabbit IgG (1:4,000; KPL Inc., Gaithersburg, MD) or horseradish peroxidase conjugated goat anti-bovine IgG (1:500) horseradish peroxidase at room temperature for 1 h, and color developed with tetramethylbenzidine (TMB) membrane substrate (KPL Inc.).

Using the primer pair *sua*-U and *sua*-D (Table 1), an approximately 4.4-kb *sua*-containing fragment was PCR amplified from genomic DNA of all strains of *S.*

Figure 1. Sequenced region of *sua*-containing DNA fragment and its adjacent gene loci. The linear transcription map was drawn based on the complete genome sequence of *Streptococcus uberis* 0140J (GenBank accession no. AM946015; <http://www.ncbi.nlm.nih.gov/genbank/>). The 4.4-kb DNA fragments (marked in gray from *sua*-U to *sua*-D) amplified by PCR with the primer pair of *sua*-D and *sua*-U from genomic DNA samples of *S. uberis* strains was sequenced and compared. nt = nucleotide.

uberis evaluated. The 4.4-kb PCR fragment contained *sua* [assigned as sub1635 in the completed *S. uberis* 0140J genome sequence; National Center for Biotechnology Information (NCBI) GenBank accession no. AM946015; <http://www.ncbi.nlm.nih.gov/genbank/>] with its flanking regions including *purR* (sub1636, DNA-binding transcriptional repressor) upstream, and *rpsL* (sub1633, 30S ribosomal subunit protein S12) and *rpsG* (sub1632, 30S ribosomal subunit protein S7) downstream (Figure 1). Sequencing of the entire PCR fragment from *S. uberis* strains showed that *sua* [2,718 nucleotides (nt)] and its flanking regions (800 nt upstream and 500 nt downstream) were highly conserved. The percentage identity of deduced AA sequences (SUAM protein) of *sua* was >97% (Table 2). Flanking regions of *sua* shared >98% identities (data not shown).

The full length of the SUAM protein was probed with rabbit anti-SUAM serum (Figure 2) and all strains of *S. uberis* evaluated produced SUAM. The small peptide PepSUAM, located at the N terminus of the mature SUAM protein, is one of the surface exposed and immunogenic regions of SUAM (Almeida et al., 2010; Luther et al., 2010). The pepSUAM sequence was identical in 19 of 20 strains of *S. uberis* evaluated (DDMTTADQSPKLQGEEA) and only 1 AA difference (underscored) existed in the remaining strain of *S. uberis* (DDMTAADQSPKLQGEEA).

Results from this investigation showed that a *sua*-containing fragment was PCR amplified from genomic DNA of all strains of *S. uberis* isolated from cows with *S. uberis* mastitis from the United States, Australia, New Zealand, and the United Kingdom. Luther et al. (2008) showed an approximately 2.6-kb *sua*-containing fragment in all strains of *S. uberis* evaluated. Differences in PCR fragment size reported in the present

study and by Luther et al. (2008) were because of the PCR primers used. Using the primer pair *sua*-U and *sua*-D, the expected PCR fragment included *sua* and its flanking genes *purR*, *rpsL*, and part of *rpsG* (Figure 2), accounting for the larger PCR fragment size (~4.4 kb) reported in this investigation. The high identities of *sua* genes with flanking regions from strains of *S. uberis* isolated from geographically diverse areas of the world suggest that SUAM may be one of the adapted mechanisms used by *S. uberis* to infect and persist in the bovine mammary gland. Based on the high level of genetic variability of different strains of *S. uberis*, it has been difficult to identify a conserved antigenic molecule in *S. uberis* (Pullinger et al., 2006). Further evaluation of SUAM as a virulence factor will facilitate delineation of the importance of this molecule as a potential vaccine target.

During the last decade, studies on host-pathogen interactions showed that SUAM may be an important virulence factor involved in adherence to and internalization of *S. uberis* into BMEC, potentially leading to persistent *S. uberis* mastitis (Almeida et al., 2006; Tamilselvam et al., 2006). Under in vitro conditions, SUAM-mediated adherence of *S. uberis* to BMEC occurred through both LF-dependent and -independent mechanisms (Patel et al., 2009). Besides facilitating bacterial colonization, SUAM-dependent adherence may trigger internalization by a caveolae-dependent intracellular trafficking pathway in mammary epithelial cells, leading to persistence of *S. uberis* intracellularly and spreading of the pathogen to deeper tissues (Almeida and Oliver, 2006; Almeida et al., 2010). The same group demonstrated that bovine serum and milk containing anti-SUAM or anti-pepSUAM antibodies significantly inhibited adherence to and internalization

Table 2. Percentage identity of deduced AA sequences of *Streptococcus uberis* adhesion molecule (SUAM) from 20 strains of *S. uberis* isolated from geographically diverse locations¹

Strain	Strain ²																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	—	98.8	98	97.8	98.9	99.2	98	98.5	98	98	97.5	98.1	98.9	99	97.1	98.1	98.6	98.6	98.3	98.5
2	1.1	—	98	97.8	98.7	98.8	98.2	98.2	98.2	98	97.6	98.1	99.8	98.8	97.5	98.1	98.3	99.7	98.1	98.2
3	2.2	2.2	—	98.9	97.8	98.1	99.1	97.4	99.1	99	97	97	97.8	97.5	97.4	97	97.5	97.5	97.2	97.4
4	2.1	2.1	1	—	97.9	98.2	99.4	97.5	99.4	100	97.1	97.1	97.9	97.6	97.2	97.1	97.6	97.6	97.4	97.5
5	1	1.2	2.1	2	—	98.9	97.9	99.2	97.9	98	98.1	98.9	98.8	98.7	98	98.9	99.3	98.5	99.1	99.2
6	0.7	1.1	1.8	1.7	1	—	98.5	98.5	98.5	98	97.8	98.1	98.9	99	97.6	98.1	98.6	98.8	98.3	98.5
7	1.9	1.7	0.8	0.4	2	1.5	—	97.5	99.9	99	97.1	97.1	98.3	97.8	97.2	97.1	97.6	98	97.4	97.5
8	1.5	1.7	2.6	2.5	0.7	1.5	2.5	—	97.5	97	98.3	99.4	98.3	98.7	97.5	99.2	99.8	98	99.8	99.9
9	1.9	1.7	0.8	0.4	2	1.5	0	2.5	—	99	97.1	97.1	98.3	97.8	97.2	97.1	97.6	98	97.4	97.5
10	2.2	2.2	0.7	0.3	2.1	1.8	0.8	2.6	0.8	—	97	97	97.8	97.5	97.4	97	97.5	97.5	97.2	97.4
11	2.5	2.4	2.9	2.8	1.8	2.1	2.8	1.6	2.8	2.9	—	98.5	97.7	97.6	97.2	98.7	98.2	97.4	98.2	98.3
12	1.8	1.8	2.9	2.8	1	1.8	2.8	0.4	2.8	2.9	1.5	—	98.2	98.6	97.4	99.4	99.3	97.9	99.3	99.4
13	1	0.1	2.1	2	1.1	1	1.6	1.6	1.6	2.1	2.2	1.7	—	98.9	97.6	98.2	98.5	99.6	98.2	98.3
14	0.9	1.1	2.5	2.4	1.2	0.9	2.1	1.2	2.1	2.5	2.4	1.3	1	—	97.1	98.3	98.8	98.6	98.6	98.7
15	2.8	2.5	2.6	2.7	1.9	2.4	2.7	2.5	2.7	2.6	2.7	2.6	2.4	2.8	—	97.4	97.6	97.2	97.4	97.5
16	1.8	1.8	2.9	2.8	1	1.8	2.8	0.7	2.8	2.9	1.2	0.4	1.7	1.6	2.6	—	99.1	97.9	99.1	99.2
17	1.3	1.6	2.5	2.4	0.6	1.3	2.4	0.1	2.4	2.5	1.7	0.6	1.5	1.1	2.4	0.8	—	98.1	99.7	99.8
18	1.3	0.2	2.5	2.4	1.5	1.1	1.9	1.9	1.9	2.5	2.6	2	0.3	1.3	2.7	2	1.8	—	97.9	98
19	1.6	1.8	2.7	2.6	0.8	1.6	2.6	0.1	2.6	2.7	1.7	0.6	1.7	1.3	2.6	0.8	0.2	2	—	99.8
20	1.5	1.7	2.6	2.5	0.7	1.5	2.5	0	2.5	2.6	1.6	0.4	1.6	1.2	2.5	0.7	0.1	1.9	0.1	—

¹Percentage divergence in lower square; percentage similarity in upper square.

²1 = O140J (United Kingdom); 2 = UT888 (United States); 3 = UT366 (United States); 4 = UT102 (United States); 5 = ATCC 13387 (United States); 6 = UTM413 (United States); 7 = C2507 (United States); 8 = NZ1636H (New Zealand); 9 = W11020 (United States); 10 = ATCC 27958 (United States); 11 = PN09-2484-2 (New Zealand); 12 = PN09-2484-3 (New Zealand); 13 = AU09-03953-4 (New Zealand); 14 = AU-03953-5 (New Zealand); 15 = PN09-02978-6 (New Zealand); 16 = PN09-02978-7 (New Zealand); 17 = VD4208 (Australia); 18 = VD5239 (Australia); 19 = VD4325 (Australia); 20 = VD5586 (Australia).

Figure 2. Western blot detecting *Streptococcus uberis* adhesion molecule (SUAM) from strains of *S. uberis*. Lane 1 = UT888; lane 2 = PN09-02183; lane 3 = PN09-2484-2; lane 4 = PN09-2484-3; lane 5 = AU09-03953-4; lane 6 = AU09-03953-5; lane 7 = PN09-02978-6; lane 8 = PN09-02978-7; lane 9 = CH09-05267; lane 10 = 0140J; lane 11 = VD4208; lane 12 = VD5239; lane 13 = VD4325; lane 14 = VD5586; lane 15 = VD4433; lane 16 = VD4203; lane 17 = VD5181. *Streptococcus uberis* strain in lane 1 was isolated in the United States, strains in lanes 2 to 9 were isolated from New Zealand, the strain in lane 10 was isolated from the United Kingdom, and those in lanes 11 to 17 were isolated from dairy cows with mastitis in Australia.

of *S. uberis* into BMEC (Almeida et al., 2006). This observation was further confirmed by a study using a *sua*-gene deletion mutant strain of *S. uberis* UT888 (Chen et al., 2011).

Using 3 different prediction methods, the N-terminal pepSUAM (also designated as SUAM peptide I), 3 other regions in the middle portion of SUAM [peptide II (AA 342–356), peptide III (AA 357–371), and peptide IV (AA 439–453)], and a region at the C-terminus of SUAM [peptide V (AA 705–719)] were predicted to be surfaced exposed B-cell epitopes (Almeida et al., 2011; Luther et al., 2010). Sequence data from this study confirmed >93% identity in those epitopes among strains of *S. uberis* evaluated (data not shown). Further study of those regions may help to identify a SUAM-based strategy for the control of *S. uberis* mastitis in dairy cows.

In conclusion, results from this investigation demonstrated that a *sua*-containing fragment was PCR amplified from genomic DNA of all strains of *S. uberis* isolated from cows with *S. uberis* mastitis from the United States, Australia, New Zealand, and the United Kingdom. All of the strains evaluated produced SUAM, demonstrating that SUAM is ubiquitous and immunogenic in all strains of *S. uberis* evaluated thus far. The suggested putative role of SUAM in the pathogenesis of *S. uberis* mastitis together with the ubiquitous presence of SUAM in strains of *S. uberis* makes SUAM a promising candidate to be considered in the development of a vaccine for the control of *S. uberis* mastitis in dairy cows.

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