Comparative molecular analysis of ovine and bovine *Streptococcus uberis* isolates

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ABSTRACT

Streptococcus uberis causes clinical and subclinical mastitis in cattle and sheep, but it is unknown whether the composition of *Strep. uberis* populations differs between host species. To address this, we characterized a collection of bovine and ovine *Strep. uberis* isolates with shared geographical and temporal origins by means of an expanded multilocus sequence typing scheme. Among 14 ovine and 35 bovine isolates, 35 allelic profiles were detected. Each allelic profile was associated with a single host species and all but one were new to the multilocus sequence typing database. The median number of new alleles per isolate was higher for ovine isolates than for bovine isolates. None of the ovine isolates belonged to the global clonal complexes 5 or 143, which are commonly associated with bovine mastitis and which have a wide geographical distribution. Ovine isolates also differed from bovine isolates in carriage of plasminogen activator genes, with significantly higher prevalence of *pauB* in ovine isolates. Isolates that were negative for *yqiL*, one of the targets of multilocus sequence typing, were found among ovine and bovine isolates and were not associated with a specific sequence type or global clonal complex. One bovine isolate carried a *gapC* allele that was probably acquired through lateral gene transfer, most likely from *Streptococcus salivarius*. We conclude that ovine isolates are distinct from bovine isolates of *Strep. uberis*, and that recombination between isolates from different host species or bacterial species could contribute to changes in virulence gene profiles with relevance for vaccine development.

Key words: *Streptococcus uberis*, molecular typing, ovine, bovine

INTRODUCTION

Streptococcus uberis is an important mastitis-causing pathogen in dairy cattle in many parts of the world, including Australia and New Zealand (McDougall et al., 2004; Shum et al., 2009), North America (Rossitto et al., 2002; Olde Riekerink et al., 2008), and Europe (Bradley et al., 2007; Sampimon et al., 2009). Multilocus sequence typing (MLST) has been used to explore the population structure of *Strep. uberis* from different countries and to identify groups of genetically related sequence types (ST), called clonal complexes (CC) or, if they are very large, global clonal complexes (GCC; Pullinger et al., 2006; Rato et al., 2008; Tomita et al., 2008). In addition, the relationship between ST or CC and sources or clinical manifestations of *Strep. uberis* mastitis has been investigated (López-Benavides et al., 2007; Pullinger et al., 2007; Tomita et al., 2008). In the United Kingdom, GCC5 is the most common group of *Strep. uberis* strains isolated from milk, whereas in New Zealand, GCC143 is the most common group, but members of both GCC occur in multiple countries (Pullinger et al., 2006). Global clonal complexes 5, 86, and 143 have been associated with clinical mastitis, latent infection, and subclinical mastitis, respectively (Tomita et al., 2008), but there does not appear to be an association between ST or CC and duration of infection (Pullinger et al., 2007). In addition to the dominant CC, a large number of singletons exist; that is, ST that differ from all other ST at more than one locus. The high level of heterogeneity in the *Strep. uberis* population is partly attributed to its recombinatorial population structure (Zadoks et al., 2005; Coffey et al., 2006; Lang et al., 2009).

Streptococcus uberis may also cause clinical or subclinical mastitis in sheep (Zadoks, 2007). In some studies from the United States and the United Kingdom, detection of *Strep. uberis* in ovine milk samples was rare and not associated with an increase in SCC (Kirk et al., 1980; Hariharan et al., 2004). In a study from Turkey, however, *Strep. uberis* was detected in 7% of udder halves from mastitic sheep, making it the third

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most common pathogen isolated from sheep udders at slaughter (Gülcü and Öngör, 2002). It was also detected in 10% of 1,186 culture-positive samples from sheep in Italy, and detection of streptococci was associated with a significant increase in SCC compared with detection of staphylococci (Cuccuru et al., 2011). Whole-genome and MLST studies of *Staphylococcus aureus* from cattle and sheep have led to identification of strains that are specifically associated with one host species (Ben Zakour et al., 2008; Vautor et al., 2009). In contrast, molecular analyses of *Strep. uberis* isolates associated with ovine mastitis have not been conducted, and there is a lack of knowledge regarding the relationship between *Strep. uberis* from ovine and bovine populations. To gain insight into the population structure of ovine *Strep. uberis*, we conducted comparative molecular analysis of a collection of *Strep. uberis* field isolates from cases of ovine and bovine mastitis with a shared geographical and temporal origin. In addition to MLST based on housekeeping genes (Coffey et al., 2006), we used analysis of the genes encoding plasminogen activator A (**PauA**) and glyceraldehyde-3-phosphate dehydrogenase (**GapC**). These targets have been associated with *Strep. uberis* virulence as well as efforts to develop mastitis vaccines (Leigh et al., 1999; Fontaine et al., 2002), and therefore may have specific clinical relevance.

MATERIALS AND METHODS

Bacterial Isolates and DNA Preparation

Streptococcus uberis isolates for MLST were kindly provided in pure culture by Francesco Cancellotti (Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy), and comprised 49 isolates from cases of ovine ($n = 14$) and bovine ($n = 35$) mastitis (clinical or subclinical) collected in 2006 or 2007 in the Lazio province of Italy. Upon receipt, strains were grown in brain heart infusion broth (Oxoid, Basingstoke, UK) and cultivated statically at 37°C for 20 to 24 h. Culture purity and colony morphology was examined on blood agar plates, prepared with blood agar base (Oxoid) supplemented with 5% sheep blood. Species identity was confirmed using the API 20 Strep kit (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's guidelines, and species-specific 16S rRNA PCR (Hassan et al., 2001).

Genomic DNA was extracted using the NucleoSpin Tissue Kit (Machery Nagel, Düren, Germany) following the manufacturer's guidelines, with minor modifications: *Strep. uberis* was cultured in 2 mL of brain heart infusion broth for 20 to 24 h to obtain stationary-phase cultures. Cultures were centrifuged for 15 min at 2,000

$\times g$, and cell pellets were resuspended in 300 μ L of gram-positive lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% Triton X 100, and 2 mg/mL lysozyme). Before addition of 2.5 mg/mL proteinase K, 100 μ L of lysate was discarded to avoid overloading the NucleoSpin columns. Genomic DNA was eluted in 60 μ L of prewarmed buffer BE containing 20 μ g/mL RNaseA, quantified using a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE), and analyzed by agarose gel electrophoresis. Samples were diluted to 100 ng/ μ L for use in PCR.

PCR and DNA Sequencing

A 7-gene MLST panel (Coffey et al., 2006) was used to determine the population structure of the isolates included in this study. Amplification of the *tdk* region using the *tdk* primers described by Coffey and coworkers (2006) failed to identify a single, clear PCR product. The published *Strep. uberis* 0140J genome sequence (GenBank accession number NC_012004; http://www.ncbi.nlm.nih.gov/nuccore/NC_012004.1; Ward et al., 2009) was therefore used as a template for designing new *tdk*-specific primers (Table 1). Analysis of 2 additional genes (*pauA* and *gapC*) was conducted using PCR primers described as part of an alternative MLST scheme (Zadoks et al., 2005). Where PCR failed to amplify a product from *pauA* using the primers described by Zadoks and colleagues (2005), alternative primers (ER45 and ER46), which included the *pauA* intergenic region, were used. Sequencing of the PCR product derived from *pauA*-negative isolates using the ER45 and ER46 primers permitted the design of additional primers (*pauB* F and R) to sequence a larger region of the gene. Details of primers used and conditions required for amplification are listed in Table 1.

The PCR products were purified using the MinElute 96 UF PCR Purification kit (Qiagen, Sussex, UK) according to the manufacturer's protocol and eluted in 30 μ L of sterile molecular biology-grade water. Sequencing was conducted in both directions by commercial companies, using the same primers as used for PCR.

Molecular and Data Analysis

Forward and reverse sequences were aligned using Clone Manager Professional Suite v. 9 (Scientific and Educational Software, Cary, NC) and the sequence files converted to FASTA format. Using SeqMan (DNASTar Inc., Madison, WI), sequences were trimmed to correspond with alleles from the *Strep. uberis* PubMLST database (<http://pubmlst.org/suberis/>), and sequences were compared against all existing allelic types from the database. When no match to previous entries was

Table 1. Oligonucleotide primer sequences and amplification conditions used for PCR and sequencing

Locus	Forward (F) and reverse (R) primer sequence (5' to 3')	Amplicon size (bp)	T _a (°C)	Reference
<i>arcC</i>	F GTT TGT GAC GCA AAA TCT TTA TCG ATA ACA R ACT CAT GGT AAC GGA CCA CAA GTT GGT AAC	518	56 ¹	Coffey et al., 2006
<i>ddl</i>	F GTC TAT ATT GAA GGT AAT GAC TTG GAA GAC TGT R TAC ATG GAC CAC TGA GTG AAT CCA GGC ATA GTA TTC	503	60 ¹	Coffey et al., 2006
<i>gapC</i>	F TTG GTA TTA ACG GTT TCG GTC R CAA GTT GAG CAG TGT AAG ACA TTT C	906	50 ²	Zadoks et al., 2005
<i>gki</i>	F GAC CGG ACC CAA AAC ACA GTC ACA GGT GCT TTT R AAG AGA ATC TGG ATT TAG GAT ATT TGA AAT ATT	564	56 ¹	Coffey et al., 2006
<i>recP</i>	F AAT TCA GGT CAC CCT GGC TTA CCA ATG GGT GCA GCC R TGT GAA AGC CAT TGA TGT TGG ACC ATC AAG TGA AAT	531	60 ¹	Coffey et al., 2006
<i>tdk</i>	F TGA CTA TTG AAA CCG CTA TTA TG R AAT GTT TAC GAC AAA CTG GAA TG	761	57 ¹	This study
<i>tpi</i>	F GTT ATT GGT CAT TCA GAA CGT CGT GAT TAC TTC R GTC AAG TAA TGC TAA GAA GCT ATC TGC TTC AAG TGA	471	60 ¹	Coffey et al., 2006
<i>yqiL</i>	F TTT CTT CTT TGA AAC GAT TAT TTT TAA GTG CTT CAG R CAA GCT CTA AGA ACA CCA ATT GGT GCA TTC GGA GGA	574	56 ¹	Coffey et al., 2006
<i>pauA</i>	F TTC ACT GCT GTT ACA TAA CTT TGT G R CCT TTG AAA GTG ATG CTC GTG	976	50 ²	Zadoks et al., 2005
<i>ER45</i> and <i>ER46</i>	F GAG ATT CCT CTC TAG ATA TCA R GGG CTG CAG ATC CGT TAA AAA ATG ACA TTA ATA T			Ward and Leigh, 2002
<i>pauB</i>	F CGG CTA GCT AGA ATA AGG GAG R GAT ATT GAT TGA GGA TAC TTT AAG CGG			This study

¹PCR conditions were 5 min at 94°C, 40 cycles of [30 s at 94°C, 30 s at annealing temperature, T_a, and 45 s at 72°C], and 7 min at 72°C.

²PCR conditions were 5 min at 94°C, 40 cycles of [30 s at 94°C, 60 s at T_a, and 45 s at 72°C], and 7 min at 72°C.

found, the original trace files were sent to the database curator for assignment of new alleles. Allelic profiles (AP) were obtained and compared with the PubMLST database; when these profiles were novel, they were assigned new ST by the database curator if sequence data were available for 7 alleles. Allele numbers for *gapC* and *pauA* were assigned in accordance with the in-house database for those genes.

Associations between binary variables; for example, host species (ovine vs. bovine) and presence or absence of specific PCR products, were explored using 2 × 2 tables and the Fisher exact test. Associations between binary variables, such as host species, and nonbinary variables, such as number of new alleles, were tested using the Wilcoxon rank sum test. Analyses were conducted with 3 sets of input data: (1) at isolate level (n = 49); (2) at nonduplicate level (i.e., if multiple isolates shared the same AP and origin, this combination was only included once but if multiple isolates with the same AP came from different origins, one representative from each origin was included; n = 40); (3) at AP level (n = 35). Statistical analyses were conducted using Statistix 9.0 (Analytical Software, Tallahassee, FL). Because of the limited number of ovine isolates, statistical significance was declared at *P* < 0.10.

Relationships between ST from the current study and the PubMLST database were explored using eBURST V3 (<http://eburst.mlst.net/>), with grouping of ST that shared at least 6 of 7 alleles. The origin of isolates that

shared ST or eBURST groups with isolates from the current study was obtained from the isolate database for *Strep. uberis* (<http://pubmlst.org/suberis/>; database last updated April 15, 2010, last accessed January 4, 2012).

RESULTS

Host Association of Allelic Profiles

Among 14 ovine and 35 bovine isolates, 35 AP were detected. Most AP were represented by a single isolate. Of 9 AP that were represented by multiple isolates, 5 originated from a single region and 4 originated from multiple regions (Table 2). Each of the 5 AP that originated from a single region was included once in statistical analyses at the nonduplicate level, whereas AP that originated from multiple regions were included twice or 3 times, depending on the number of regions of origin. Each AP was associated with a single host species (Table 2). With the exception of one isolate, all isolates belonged to AP that were new to the *Strep. uberis* MLST database (Table 2). Of 34 new AP, 10 were the result of new combinations of existing alleles, and 24 AP included alleles that were new to the database. The number of new alleles per ST ranged from 1 to 4, with a median of 2, and was higher for ovine than for bovine isolates (Wilcoxon rank sum test; *P* < 0.10 at isolate, nonduplicate, and AP levels).

Table 2. Allelic profiles of ovine and bovine *Streptococcus uberis* isolates from this study¹

Host/isolate	Allele										ST	eBURST group ²	Origin ³
	<i>arcC</i>	<i>ddl</i>	<i>gki</i>	<i>recP</i>	<i>tdk</i>	<i>tpi</i>	<i>yqiL</i>	<i>pauA</i>	<i>pauB</i>	<i>gapC</i>			
Ovine													
1	5	1	4	3	28	1	3	9	0	7	292	6	RM1
2, 8	21	30	30	2	44	4	28	0	1	30	293	Singleton	VT1
3	10	1	5	2	45	4	3	0	1	31	294	Singleton	RM2
4	12	2	5	3	20	1	3	1	0	7	295	Singleton	RM3
5	41	1	5	2	46	4	31	1	0	27	296	Singleton	RM4
7	10	30	31	1	17	4	33	0	2	27	297	Singleton	Unknown
9	5	1	4	3	28	4	2	9	0	7	298	Singleton	VT1
10, 11	5	4	4	3	28	4	29	9	0	7	299	Singleton	RM1
12	39	1	5	2	46	4	32	0	1	27	300	Singleton	RM5
13	42	2	32	2	47	4	15	1	0	27	301	Singleton	RM6
6	21	30	16	2	59	1	28	36	0	3	377	Singleton	RM7
14	10	1	33	2	53	4	0	1	0	28	U1	Singleton	RM5
Bovine													
39	3	1	4	4	3	2	3	9	0	4	30	4	RM8
15	9	28	3	4	3	1	3	1	0	12	302	Singleton	RM9
16	2	1	4	2	10	1	3	23	0	4	303	9	VT2
26	2	1	4	2	49	1	3	23	0	4	304	9	RM10
17	2	1	4	2	49	1	3	23	0	4	304	9	RM11
37	42	6	5	2	48	14	3	6	0	33	305	Singleton	RM12
18	42	6	5	2	48	14	3	6	0	33	305	Singleton	RM13
36, 40, 47	42	6	5	2	48	14	3	6	0	33	305	Singleton	RM14
19	2	3	4	2	3	2	3	23	0	19	306	Singleton	RM15
48	1	1	4	1	2	1	3	4	0	8	307	1	RM13
20	1	1	4	1	2	1	3	4	0	8	307	1	FR1
21	40	1	4	2	49	1	3	6	0	8	308	9	RM9
22	10	30	3	3	5	1	3	9	0	7	309	19	VT3
23	4	3	29	2	3	1	15	0	3	1	310	Singleton	VT3
24	10	30	3	3	5	4	3	9	0	32	311	19	VT3
25, 46	4	1	5	2	2	4	17	6	0	8	312	Singleton	RM16
27	3	1	5	2	50	1	3	6	0	8	313	1	RM17
28	2	3	5	1	2	1	3	4	0	21	314	Singleton	RM17
29	4	31	5	2	51	4	15	0	4	1	315	Singleton	RI1
30, 31	2	1	4	1	2	1	3	23	0	4	316	1	RM18
32	2	30	4	2	3	4	30	0	3	34	317	Singleton	FR2
35, 38	42	1	5	2	10	1	3	4	0	4	318	1	RM14
45	42	1	5	2	10	1	3	4	0	4	318	1	RM19
41, 42, 43	5	15	5	2	2	1	3	9	0	1	319	Singleton	RM20
44	42	1	5	2	50	4	17	6	0	4	320	Singleton	RM21
50	2	5	4	2	47	4	10	37	0	34	321	Singleton	LT1
34	4	29	34	4	52	15	0	1	0	3	U2	Singleton	RM9
49	4	30	5	2	29	4	0	1	0	1	U3	Singleton	VT4

¹New alleles are shown in bold face. Sequence types (ST) were assigned to isolates that had completed allelic profiles for *arcC*, *ddl*, *gki*, *recP*, *tdk*, *tpi*, and *yqiL* and are otherwise listed as unknown (U). A value of zero indicates absence of PCR product for the target gene.

²Based on eBURST (<http://eburst.mlst.net/>) analysis using similarity at 6 of 7 loci as grouping criterion, and using PubMLST profiles database (<http://pubmlst.org/suberis/>) from July 20, 2011. eBURST groups may change in size, composition, and number assigned to the group as the database evolves.

³Letters indicate province in the Lazio region of Italy: FR = Frosinone, LT = Latina, RI = Rieti, RM = Roma, and VT = Viterbo; numbers indicate individual farms or veterinary practices.

Host Association of Plasminogen Activator Alleles

Isolates that belonged to the same AP also shared the same *pauA* or *pauB* and *gapC* alleles (Table 2). Allele *pauB* could be amplified from all isolates that failed to yield a *pauA* PCR product ($n = 7$). Presence of *pauB* was more common among ovine isolates than among bovine isolates. The difference was significant at the isolate level, but not at the nonduplicate or AP

levels (Wilcoxon rank sum test; $P = 0.03$, $P = 0.10$, and $P = 0.16$, respectively). Four *pauB* alleles were detected (Figure 1); *pauB* allele 1 was only detected in sheep, but it was associated with multiple AP and geographic origins, whereas *pauB* allele 3 was only detected in cattle, and was associated with multiple AP and geographic origins (Table 2). Isolates with *pauB* had significantly more new alleles than isolates with *pauA* (Wilcoxon rank sum test; $P < 0.03$ at the iso-

late, nonduplicate, and AP levels). All *pauA*-negative isolates differed at 4 or more alleles from previously published *pauA*-negative isolates (Lang et al., 2009).

Isolates Negative for *yqiL*

Of 49 isolates, 3 were negative for *yqiL*; the *yqiL*-negative isolates included 1 ovine and 2 bovine isolates, which belonged to 3 unrelated AP and came from 3 different geographic origins. All *yqiL*-negative isolates were positive for *pauA* (Table 2). In the online database, ST are not assigned to isolates that are *yqiL*-negative, hence the use of AP rather than ST to allow for description of all isolates in the current study. The *yqiL*-negative isolates differed from all ST in the online database in 3 or more alleles, counting absence versus presence of *yqiL* as an allelic difference. All *yqiL*-negative isolates from the current study differed from previously described *yqiL*-negative isolates in at least 4 alleles (Tomita et al., 2008; Table 3).

Host Association and Geographic Origin of eBURST Groups

One ovine isolate and 15 bovine isolates belonged to ST that were part of an eBURST group; that is, a group of ST that were single locus variants (SLV) of each other. The remaining isolates belonged to singletons (Table 2). Ovine isolates were significantly more likely than bovine isolates to belong to singletons (Fisher Exact; $P < 0.05$ at the isolate, nonduplicate, and ST levels). The most common eBURST group was group 1, which includes GCC5 and GCC143. Eight bovine isolates belonged to this group, but none of the ovine isolates did (Table 2). The difference was significant (Fisher Exact; $P < 0.10$) at the isolate and nonduplicate levels, but not at the ST level.

The only ovine isolate that was part of an eBURST group belonged to ST292 and eBURST group 6 (Figure 2). The members of this group originate from Sweden, Italy, and the UK. Sequence type 292 is the only member of the group to have been isolated from ovine rather than bovine milk. One bovine isolate belonged to ST30, which is at the core of eBURST group 4. This group includes multiple ST from multiple countries (Figure 2). Two bovine isolates belonged to eBURST group 9, which consists of 2 ST from the current study that are SLV of each other; and 2 isolates belonged to eBURST group 19, which also consists of 2 ST from the current study that are SLV of each other. Three bovine ST from the current study—ST303, ST318, and ST319—had previously been identified in Italy (www.pubmlst.net/suberis). Isolates with these ST originated from 1, 3, and 1 Italian dairy herd(s), respectively, and all herds

were unrelated to the flocks and herds involved in the current study (Paolo Moroni, Università degli Studi di Milano, Milan, Italy; personal communication).

Diversity of Glyceraldehyde-3-Phosphate Dehydrogenase

The majority of *gapC* alleles from Italian isolates were previously described or diverged from existing *gapC* alleles by 0.1 to 0.9% (sequence heterogeneity). One bovine isolate (isolate 24; Table 2) had a *gapC* allele that diverged from all other alleles by 15.3 to 16.1%, which is more than the average difference (10%) between the *gapC* allele from *Streptococcus parauberis* (Zadoks et al., 2005) and *gapC* alleles of *Strep. uberis*. BLAST analysis of the divergent *gapC* allele revealed 99.9% identity (840 of 841 nucleotides) with the *gapC* allele of *Streptococcus salivarius* (CP00288.1 and FR873481.1), whereas the next best matches showed 91% or less identity. The isolate with the highly divergent *gapC* allele was an SLV of another isolate in the current study.

DISCUSSION

To our knowledge, there has been no previous investigation of *Strep. uberis* isolated from cases of ovine mastitis. Despite the observed heterogeneity of the bovine-derived *Strep. uberis* population, the ovine isolates described in this study were recognizably distinct from bovine isolates obtained from the same region during the same sampling period. Ovine isolates contained a larger median number of new alleles per AP, were more likely to belong to singletons, and less likely to be related to GCC5 and GCC143 than bovine isolates. Taken together, these data would suggest that different subpopulations of *Strep. uberis* might be better adapted to cause mastitis in cattle or sheep. Although gastrointestinal carriage of *Strep. uberis* in cattle has been described, no such data are available for sheep (Zadoks, 2007). Consequently, it is not possible to state whether differences between ovine and bovine mastitis-causing strains result from initial adaptation to a primary niche (i.e., the gastrointestinal tract), a subsequent adaptation to the mammary gland environment, or both. The existence of apparently distinct *Strep. uberis* populations in cows and sheep suggests that mastitis arises as a result of infection from the host's resident population rather than from environmental sources that are common to both host species.

Considering the heterogeneity of the global *Strep. uberis* population, any MLST-based study of *Strep. uberis* is likely to yield new ST. Some ST are over-represented in specific geographic areas; for example,

pauB SK880	121	tttactgtcctcatcttttattggaattgttggaactactgggaattccatcacaaagtagaggtatcacttcaaaagaagttaattaccaagaaccacaagggactatgttaacgataaat
I2 pauB	1	-----
I3 pauB	1	-----
I7 pauB	1	-----
I8 pauB	1	-----
I12 pauB	1	-----
I23 pauB	1	-----
I29 pauB	1	-----
I32 pauB	1	-----
pauB SK880	241	attaccgggagaagcaaggaggagacagttgctcctttctccagaatatatgcaatttcgcttaacagcgggagaagttattaataaaaaagagttactggataaagtgcaactgtgtata
I2 pauB	104	-----
I3 pauB	104	-----
I7 pauB	104	-----
I8 pauB	104	-----
I12 pauB	104	-----
I23 pauB	104	-----
I29 pauB	104	-----
I32 pauB	104	-----
pauB SK880	361	gacagtgctgcttcttaaccagtttgaagttgttgatttcaaacccgaatcaaaaggtggaatggtcotattttgacgcgcacgaattgcttgatctactgaagaggatttatcgtaact
I2 pauB	224	-----
I3 pauB	224	-----
I7 pauB	224	-----
I8 pauB	224	-----
I12 pauB	224	-----
I23 pauB	224	-----
I29 pauB	224	-----
I32 pauB	224	-----
pauB SK880	481	gattattcaatttatgaaaagaacacttctattctcttaacaggccctgttattattccagagaaaaagttccaacacagagaaaaaactattgatagtaacacaggtgctcattctctaga
I2 pauB	344	-----
I3 pauB	344	-----
I7 pauB	344	-----
I8 pauB	344	-----
I12 pauB	344	-----
I23 pauB	344	-----
I29 pauB	344	-----
I32 pauB	344	-----
pauB SK880	601	cacagcgttctctttatgaaaagaacggcgaatcaatgaattctgttccacaactacacagctatcttctatttctaaactgcaaaacctgttcgtcaattcaatcagaagaactc
I2 pauB	464	-----
I3 pauB	464	-----
I7 pauB	464	-----
I8 pauB	464	-----
I12 pauB	464	-----
I23 pauB	464	-----
I29 pauB	464	-----
I32 pauB	464	-----
pauB SK880	721	tatcaagctgctcaatcactttttgagcaacaaaagaatatcatgaaggttatcaattaatcaaacggttgaacacatcgatttcagaaaatgaaaagcctatcgcgattgtttatcaa
I2 pauB	584	-----
I3 pauB	584	-----
I7 pauB	584	-----
I8 pauB	584	-----
I12 pauB	584	-----
I23 pauB	584	-----
I29 pauB	584	-----
I32 pauB	584	-----
pauB SK880	841	tttcaaaacggaaaaccccttcaactatacagatttcacaactaagagaggtttcaaatgggattattcacaa-----gcaattagagacgatattcattgagaactattac
I2 pauB	704	-----
I3 pauB	704	-----
I7 pauB	704	-----
I8 pauB	704	-----
I12 pauB	704	-----
I23 pauB	704	-----
I29 pauB	704	-----
I32 pauB	704	-----
pauB SK880	946	atttcaaaaatggcgatgactattaccctgaacaaatccaattacgcttgatttgacgaatgaagaagaacaagtagatcgtactttaactttagatttgcataaagaacacacatt
I2 pauB	809	-----
I3 pauB	809	-----
I7 pauB	824	-----
I8 pauB	809	-----
I12 pauB	809	-----
I23 pauB	809	-----
I29 pauB	809	-----
I32 pauB	809	-----
pauB SK880	1066	gagcatattaaacactatcttagtgaagcagatctcactgaattgatgctaaaaacaggagatcattatttggtttagtcagaatgtcaagcaggtatcatccaccatttccaggctaac
I2 pauB	929	-----
I3 pauB	929	-----
I7 pauB	944	-----
I8 pauB	929	-----
I12 pauB	929	-----
I23 pauB	929	-----
I29 pauB	929	-----
I32 pauB	929	-----
pauB SK880	1186	tatttcagcaaaagaaactctgatttcttactataacccgattcagcttgcaataatgggtactgaataaagtgaacgaagactaacaggtctctggtttttgcttctcattttgat
I2 pauB	1049	-----
I3 pauB	1049	-----
I7 pauB	1064	-----
I8 pauB	1049	-----
I12 pauB	1049	-----
I23 pauB	1049	-----
I29 pauB	1049	-----
I32 pauB	1049	-----

Figure 1. Alignment of novel *pauB* sequences from *Streptococcus uberis* with reference sequence AJ314852. Isolates I2, I3, I7, I8, and I12 originate from ovine milk; isolates I23, I29, and I32 originate from bovine milk. Nucleotide polymorphisms are highlighted.

Table 3. Comparison of allelic profiles for ovine and bovine *yqiL*-negative isolates of *Streptococcus uberis*

Host species	Allele						Country	Reference
	<i>arcC</i>	<i>ddl</i>	<i>gki</i>	<i>recP</i>	<i>tdk</i>	<i>tpi</i>		
Ovine	10	1	33	2	53	4	Italy	This study
Bovine	4	29	34	4	52	15	Italy	This study
Bovine	4	30	5	2	29	4	Italy	This study
Bovine	1	1	3	2	1	1	Australia	Tomita et al., 2008
Bovine	3	2	28	2	6	4	Australia	Tomita et al., 2008
Bovine	6	14	4	15	22	13	Australia	Tomita et al., 2008
Bovine	21	21	18	11	41	6	Australia	Tomita et al., 2008

members of GCC5 in the UK and members of GCC143 in New Zealand. These associations, however, are not absolute because members of GCC5, GCC86, and GCC143 have been observed in the UK, Portugal, Australia, and New Zealand (Rato et al., 2008; Zadoks et al., 2011). New ST in the current study, which is the first to describe *Strep. uberis* isolates from Italy, also included members from GCC5, GCC86, and GCC143. In addition, several ST, including the newly identified ST292, were members of smaller CC that included isolates from several European countries, including the UK, continental Europe, and southern as well as northern Europe. Apparent geographic clustering of ST may be a function of sample size and bacterial population heterogeneity rather than an indication of the existence of independently evolving populations. It will be interesting to see whether the apparent divergence of bovine and ovine isolates continues to be observed when larger collections of ovine isolates are identified. The fact that this divergence was observed among temporally matched isolates from a geographically limited

area suggests that it may reflect a biological difference rather than being due to study design or sample size. Experimental studies using isolates of ovine and bovine origin to challenge cattle and sheep might provide further insight into potential biological differences.

Ovine *Strep. uberis* isolates differed from bovine isolates in the plasminogen activator-encoding gene that they carried; that is, they were more likely than bovine isolates to contain the alternative plasminogen activator *pauB* rather than the more common plasminogen activator *pauA*. Where *pauB* was present, alleles appeared to be host species-associated, although we recognize that the number of isolates per allele is currently too small to detect significant associations. Detection of *pauB* has been described previously in a bovine *Strep. uberis* isolate from Denmark (Ward and Leigh, 2002), and *pauA* negative isolates have been described in multiple studies (Khan et al., 2003; Zadoks et al., 2005). It is hypothesized that plasminogen activators contribute to the acquisition of essential nutrients from milk casein, increasing the ability of bacteria to survive in nu-

Figure 2. eBURST (<http://eburst.mlst.net/>) groups 4 (left) and 6 (right), as identified based on multilocus sequence typing of *Streptococcus uberis*, with country of origin: FR = France, IT = Italy, NL = the Netherlands, SW = Sweden, UK = United Kingdom. Numbers indicate sequence types; text in italics indicates an isolate of ovine origin.

tritionally limited environments (Kitt and Leigh, 1997) and to invade tissues via the breakdown of extracellular matrix components (Jones and Holt, 2004; Sun et al., 2004). PauA and PauB have equivalent capacities to activate bovine plasminogen but PauB has an enhanced capacity to activate ovine plasminogen compared with PauA (Ward and Leigh, 2002). The observed variation in carriage of genes such as *pauA* or *pauB* highlights a potential problem with respect to the development of vaccines against *Strep. uberis*, because vaccines based on a single strain or a single antigen may not provide protection against all strains of the pathogen. Furthermore, the highly recombinatorial nature of the *Strep. uberis* population and the potential ability for gene derivatives that predominate within a given environment (e.g., sheep) to cross into another environment (e.g., cattle) mean that vaccine design may need to take into account ovine-derived *Strep. uberis* strains, which thus far have not been considered.

The genetic heterogeneity of potential vaccine candidates is also illustrated by the presence of a *gapC* allele that diverged from known *gapC* alleles in *Strep. uberis* by more than 15%, while showing 99.9% sequence identity to *gapC* of *Strep. salivarius*. Here, alleles from different bacterial species, rather than different host species, appear to contribute to this heterogeneity. Recently, the presence of the salivarin operon, which originates from *Strep. salivarius*, was described in several isolates of *Streptococcus agalactiae* derived from bovine mastitis (Richards et al., 2011), providing evidence that lateral gene transfer between *Strep. salivarius* and mastitis-causing streptococci is possible. Although *Strep. salivarius* is primarily an organism of the human oral cavity, the salivarin operon was not detected in human *Streptococcus agalactiae* isolates (Richards et al., 2011). It has been suggested that lateral gene transfer between *Strep. salivarius* and mastitis-causing streptococci may occur within the bovine environment (Richards et al., 2011), because *Strep. salivarius* has been isolated from bovine bulk tank milk and from quarter milk samples with SCC >200,000 cells/mL (Zadoks et al., 2004; Nam et al., 2009).

The typeability of the isolates included in this study was 93.9% using the Coffey-MLST scheme (Coffey et al. (2006), owing to the presence of 3 *yqiL*-negative isolates. Similarly, 4 of 46 Australian *Strep. uberis* isolates failed to yield a *yqiL* amplicon (Tomita et al., 2008). Comparison of Italian and Australian data showed that absence of a *yqiL* amplicon is not associated with any specific ST or CC. Although isolates that lack a target gene are not assigned an ST in the *Strep. uberis* PubMLST scheme, some MLST schemes do assign ST to such isolates, allowing for their inclusion in subsequent analyses and monitoring of their dissemination

(Zadoks et al., 2005; Pérez-Trallero et al., 2007). In a study of *Streptococcus pyogenes* isolates from Spain, the *yqiL*-negative ST403 was, in fact, the most prevalent clone detected. Inclusion of *yqiL*-deficient *Strep. uberis* isolates in the PubMLST scheme could be considered. Alternatively, genes that can be amplified from every isolate (e.g., *gapC*) could be used to replace *yqiL* in an MLST scheme. Although alteration of an MLST scheme would be labor intensive, precedent exists and modification of MLST schemes may result in enhanced resolution (Thomas et al., 2007) or improved understanding of the population structure of a pathogen (Sørensen et al., 2010).

CONCLUSIONS

Ovine and bovine mastitis appear to be caused by distinct subpopulations of *Strep. uberis*, with regard to both housekeeping and virulence genes. Because of the highly recombinatorial nature of *Strep. uberis*, lateral gene transfer may result in exchange of genetic material between bacterial subpopulations associated with different host species and between streptococcal species. This could affect the efficacy of *Strep. uberis* mastitis vaccines, for example, if the composition of the ovine *Strep. uberis* population is not considered during vaccine development and evaluation.

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