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A novel subtraction diversity array distinguishes between clinical and non-clinical *Streptococcus uberis* and identifies potential virulence determinants

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ABSTRACT

Streptococcus uberis is an important bovine mastitis pathogen, but not all isolates have equal capacity to cause disease. The aims of this study were to identify possible virulence-associated genes that could be used to identify isolates with enhanced virulence. DNA from a pool of putative commensals was subtracted from a clinical pool resulting in a set of DNA sequences (probes) that were enriched in the clinical mastitis group. The probes were hybridised with DNA from a collection 29 isolates from cases of clinical mastitis and isolates not associated with disease. Hybridization revealed five major clusters. The first cluster (7 isolates) consisted almost entirely of commensals, while the second (7 isolates) was mixed. The remaining three clusters contained 15 *S. uberis* isolates from cows with clinical mastitis. Twenty-six probes were selected for sequencing based on principal component analysis (PCA) or their presence mainly in clinical isolates. PCA identified five probes with clear differences in intensity between signals from clinical isolates and commensals; these probes could represent novel virulence determinants. Manual inspection of arrays identified genes prominent among clinical isolates that specify carbohydrate and lipid metabolism (possible role in the growth or survival of *S. uberis* in milk) and genes specifying hypothetical proteins, possibly novel virulence factors. The common occurrence, among clinical isolates, of probes having homology with transposases and insertion sequences suggests recent acquisition of factors that could be associated with virulence. These results suggest the existence of a subset of *S. uberis* with enhanced virulence, due possession of virulence-associated gene sequences.

1. Introduction

Mastitis is the most economically important disease of dairy cattle worldwide. *Streptococcus uberis* is one of the most important mastitis pathogens in milk-producing countries worldwide (Botrel et al., 2010; Bradley et al., 2007; Olde Reikerink et al., 2008; Shum et al., 2009); however, the factors that contribute to the virulence of this environmental pathogen are not well understood. Proposed virulence factors include molecules that promote growth in milk include a plasminogen activator (PauA) (Leigh and Lincoln, 1997; Hossain et al., 2015), an oligopeptide permease (Opp) (Smith et al., 2002), a lipoprotein responsible for the active transport of manganese (MtuA) (Smith et al., 2003), and a putative membrane-bound protein (OppA-like) that transports essential amino acids across the cytoplasmic membrane Taylor et al., 2003). Other proposed factors include an adhesion molecule (SUUM) (Almeida et al., 2006) and sortase A which anchors virulence-associated proteins at the surface of *S. uberis* (Leigh et al., 2010). The expression of many putative virulence-associated genes is co-ordinately upregulated by *vru* during growth of *S. uberis* in milk (Egan et al., 2012).

These genes, singly or as a group, have not been shown to be specifically associated with mastitis; however, there is evidence that certain host-adapted strains of *S. uberis* have enhanced ability to cause clinical mastitis (Tomita et al., 2008; Lang et al., 2009; Tassi et al., 2013). While approximately 82.5% of the genome of *S. uberis* consists of genes that are common to all strains, the remaining content is variable across different strains, and possibly related to transmissibility and host adaption (Lang et al., 2009). Moreover, certain clonal complexes (GCC 5 and GCC 143) are highly-associated with clinical and subclinical mastitis, while others (GCC 86) are associated with isolates from cows with low milk somatic cell counts (SCCs) (Tomita et al., 2008).

Although microarray technology has been used to analyse the differential expression of putative virulence associated genes (Lang et al., 2009; Egan et al., 2012), it has never been used to identify DNA fragments associated with virulence of *S. uberis* using a suppression subtractive hybridization (SSH) based approach. SSH is a DNA subtraction technique where DNA from one species/genotype can be subtracted from DNA of another species/genotype, thus theoretically enriching for

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sequences unique to the species/genotype of interest (Diatchenko et al., 1996). At RMIT University, a novel method of utilizing SSH-based DNA subtraction to enrich for sequences specific for particular species or genotypes has been recently developed. Unlike SSH-based DNA fingerprinting arrays that involve pairwise DNA subtractions between the species to be fingerprinted, the SDA involves subtraction of pooled genomic DNA from two different sets of contrasting genotypes (Mantri et al., 2012; Olarte et al., 2013a,b). The SDA technique that was originally developed to fingerprint medicinal plants belonging to flowering kingdom (angiosperms) was successfully employed to fingerprint medicinal species from Asterids clade (1/3 of flowering plants), dried herbal formulations, and chemotypes of two commercially important medicinal species (Salvia and Echinacea) (Mantri et al., 2012; Olarte et al., 2013a, b). In all these studies, the SDA not only allowed fingerprinting species and grouping them according to phylogeny or geographical origin, but also allowed discovery of novel DNA fragments that can be used for DNA barcoding or identification of genotypes with high levels of bioactives. However, this technique has never been employed in prokaryotes or to detect DNA fragments associated with virulence.

The aims of this study were to use the SDA technique to investigate (i) whether there were any differences in the range of putative virulence factor genes between isolates of *S. uberis* from cows with clinical mastitis and those with low milk SCCs and (ii) to identify novel sequences of *S. uberis* that might contribute to its ability to cause mastitis. Specifically, we used comparative genome analyses of isolates from cows with clinical mastitis and cows with low milk SCCs. DNA extracted from a pool of isolates belonging to GCC ST86 (low cell count pool) was subtracted from a clinical pool consisting of DNA from isolates belonging to GCC ST143/GCC ST5 (Tomita et al., 2008). Next, a set of 29 *S. uberis* isolates were fingerprinted to evaluate the potential of the SDA technique to discriminate between clinical mastitis and low SCC isolates, and to establish genetic relationships between these genotypes.

2. Materials and methods

2.1. S. uberis isolates

Twelve isolates of *S. uberis* were selected as the source of genomic DNA for development of tester and driver pools, based on their GCC (Tomita et al., 2008), pulsed-field gel electrophoresis (PFGE) profiles (Abureema et al., 2014) and clinical status. The tester pool consisted of 6 isolates from cows with clinically-defined mastitis, that had previously been assigned to GCC 5 or GCC 143 by multilocus sequence typing (MLST), hereafter referred to as CM, while the 6 isolates in the driver pool had no clinical evidence of mastitis, low milk SCCs and belonged to GCC 86 (hereafter referred to as LCC) (Tomita et al., 2008; Tassi et al., 2013) (Table 5). MLST sequences a small number of housekeeping (reference) genes that are essential for host survival, while our subtractive microarray flags the presence or absence of a gene fragment or major differences in total sequences between strains. While the method may also detect differences in sequences between different MLST types, most of the flagged differences will be in genes that are not essential for host survival but may enhance strain virulence. Another 29 isolates, all with different PFGE profiles (Table 1), were selected from our culture collection for hybridisation with the SDA. These included 20 isolates from cows with clinical mastitis and nine from cows with low SCCs. The majority of isolates were sourced from farms in Gippsland, Eastern Victoria, Australia, but one isolate was from the Timboon district in Western Victoria, Australia. The identification of all isolates was confirmed using selected cultural, and biochemical tests (carbohydrate fermentation, aesculin hydrolysis, and hippurate hydrolysis), serological tests (streptococcal agglutination system containing A. B, C, D, F and G group-specific antibodies) and by PCR of 23S rRNA (Hassan et al., 2001), *HasA*, *HasC* (Field et al., 2003), and *PauA* genes.

Table 1
Streptococcus uberis isolates selected as tester and driver strains.

Disease status[1]	Isolate ID	PFGE type	Global clonal complex (GCC)[2]
CM	2893-1	28	143
CM	2520-1	26	143
CM	5851	33	5
CM	2481-1	5b	5
CM	2907-1	3	5
CM	2956-4	5c	5
Co	2655-5	22	86
Co	3217-5	31b	86
Co	3327-3	32	86
Co	3217-2	18	86
Co	2690-2	27	N
Co	3147-4	11a	N

[1] CM, clinical mastitis (tester strains), clinical signs of mastitis and somatic cell count > 250 cells/mL; Co, commensal, low somatic cell count, no clinical or cytological evidence of mastitis, defined as commensal (driver strains). PFGE, pulsed field gel electrophoresis, GCC, global clonal complex.
[2] Tomita et al. (2008), N does not belong to any GCC.

2.2. DNA extraction

Genomic DNA was extracted from *S. uberis* strains using a Wizard Genomic DNA Purification Kit in accordance with the manufacturer's instructions (Promega, Madison, WI) then purified and cleaned using the DNeasy column of the DNeasy Blood & Tissue Mini Kit (Qiagen, Valencia, CA) following the protocol in the user manual. The concentration and purity of the genomic DNA pools were evaluated spectrophotometrically and DNA was stored at −20 °C until required.

2.3. Genomic DNA subtraction and library construction

The gDNA was pooled into two separate groups, CM and LCC, by mixing equal quantities of DNA from individual species belonging to that group. Subsequently, 4 µg of pooled gDNA from each group was restriction digested in a 50 µL reaction using 5U of *Hae*III and *Alu*I (New England Biolabs,). As previously described (Mantri et al., 2012), the digested LCC gDNA pool was subtracted from digested CM gDNA pool to isolate CM-specific DNA using the Clontech PCR-Select™ cDNA Subtraction Kit (Clontech, Mountain View, CA). The CM-specific DNA fragments were cloned into pGEM-T® Easy Vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI), resulting into 280 clones with inserts of 250–750 bp.

During the first hybridization of the extraction procedure, the final volume (4 µL) of each sample was heat denatured at 98 °C for 90 s using the PCR machine heat block. The samples then were allowed to anneal at 60 °C for 1.5 h. This temperature was chosen because the hybridization temperature for subtractive hybridization depends on the GC content of a particular genome. A review by Hacker et al. (1997) showed that the optimal hybridization temperature for genomic DNA with an average GC content 40–51%, for instance, *S. typhimurium, D. nodosus, E. coli,* and *Y. enterocolitica*, is 63 °C. However, in the case of *C. perfringens* (26.5%) or *S. uberis* (36.63%), the hybridization temperature might be reduced to 60 °C as the GC value of the genomic DNA is low.

2.4. CM-specific clone amplification and SDA printing

The 280 CM-specific DNA clones were amplified in 100 µL PCR reactions using Clontech nested primers as described (Mantri et al., 2012). PCR products were transferred into V-bottom polypropylene 96-well plates and purified by ethanol/sodium acetate precipitation before resuspending in 50% dimethyl sulfoxide (DMSO). The 280 CM-specific DNA fragments were spotted on a Corning GAPS II coated slides

(Corning Incorporated Life Sciences, Acton, MA) using BioRobotics MicroGrid II Compact (Genomics Solutions, Ann Arbor, MI) microarray spotter. Control spots on the SDA included printing control (Cy-3) and negative controls *viz.*, printing buffer (50% DMSO), nested primer 1 and 2R (Clontech, Mountain View, CA), and pGEM-T® Easy Vector (Promega, Madison, WI) digested with *Hae*III and *Alu*I. Positive controls consisted of three genes; *hasA*, *hasC*, and *pauA*. In a previous study in our laboratory, a statistically significant link was demonstrated between *hasA* ($P = 0.016$) and disease status, while *hasC* and *pauA* were present in all isolates from mastitis cases (clinical and subclinical) as well as cows with low SCCs (Tomita et al., 2008).

2.5. Target synthesis

The SDA was first validated by testing for the success of LCC gDNA subtraction by separately hybridizing DNA fragments from pooled CM and pooled LCC onto the array. The array was then used to test the ability to differentiate a population of 29 *Streptococcus uberis* isolates containing 20 CM and 9 LCC isolates (Table 1). The preparation of targets in all cases involved the double digestion of 0.5 µg of pooled total DNA with *Alu*I and *Hae*III, and purification using Qiaquick® PCR Purification Kit (Qiagen Inc.). Biotin-11-dUTP was then incorporated into restriction digested gDNA fragments using the Biotin DecaLabel™ DNA Labeling Kit (Fermentas, ON, Canada) following the manufacturer's guidelines except that the incubation time was increased to 20 h, then the reaction was stopped with 1 µl 0.5 M EDTA, pH 8.0 and labelled gDNA fragments were purified using Qiaquick® PCR Purification Kit.

2.6. Hybridization of the SDA

The SDA slides were pre-hybridized for 45 min at 42 °C in a pre-warmed solution containing 5X standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS), 1% bovine serum albumin (BSA) and 25% formamide. The slides were rinsed twice with sterile MilliQ water and immediately dried with an air gun.

The biotin-labelled targets (dried to 16 µL) were added to 17.5 µL of fresh 2X Hybridization buffer (250 µL of formamide, 250 µL of 10X SSC, 10 µL of 10% SDS), 0.5 µL of 5 µg/µL Human Cot1 DNA (Sigma-Aldrich, St Louis, MO), 0.5 µL of 10 mg/mL Poly A (Sigma-Aldrich) and 0.5 µL of 10 mg/mL salmon sperm DNA (Sigma-Aldrich). The mixture was denatured at 100 °C for 2 min and immediately applied onto the array under a 22 × 25-mm lifter slip (Grale Scientific, Victoria, Australia). The slides were then placed in waterproof, humidified hybridization chambers (Corning Incorporated Life Sciences, NY) and incubated overnight in a 42 °C water bath. Following hybridization, the slides were washed twice for 5 min in 500 mL Wash buffer 1 (1X SSC with 0.1% SDS), once for 5 min in 500 mL Wash buffer 3 (0.1X SSC with 0.1% SDS), and once for 5 min in 500 mL Wash buffer 4 (0.1X SSC). Subsequently the slides were transferred to 500 mL of 6X SSPE-T buffer (0.9 M NaCl, 0.06 M $NaH_2PO_4.H_2O$, 0.006 M EDTA, 0.005% Triton X-100, pH 7.4) without allowing them to dry.

The biotinylated DNA targets bound on the array were then labelled with fluorescent FluoroLink™ streptavidin-labelled Cy3 dye (Amersham Pharmacia, UK) using a biotin–streptavidin system. Briefly, 200 µL of a Detection solution (0.5 µL of 0.8 µg/µL streptavidin-labelled Cy3, 0.8 µl of 25 µg/µL BSA, made to 200 µL with 6X SSPE-T) was applied directly onto the array surface and a 22 × 25-mm lifter slip was placed over it to evenly distribute the solution on the array. The slides were placed in hybridization chambers, wrapped in aluminum foil and incubated at 37 °C for 1 h in the dark. Finally, the slides were washed thrice in 6X SSPE-T for 5 min and rinsed with sterile MilliQ water before being dried with an air gun. All hybridizations were performed with six technical replicates (corresponding to six sub-arrays) and three biological replicates, resulting in 18 data points for each array feature.

2.7. Scanning and data analysis

Slides were scanned with a ScanArray Gx (PerkinElmer Life and Analytical Sciences, Downers Grove, IL) microarray scanner in conjunction with the supplied software. The slides were scanned with a resolution of 10 µm at 532 nm (Cy3, green laser) and at 55% photomultiplicator (PMT) gain whilst keeping background noise low. The scanned array was quantified using PerkinElmer ScanArray Express software v 2.0. The program individually quantified the signal intensity at each probe and normalized the data using the adaptive circle and LOWESS functions. Probes which did not hybridize were automatically flagged by the scanning software and labelled as 'Bad'. Manual flagging was used to remove spots displaying inconsistent hybridization such as 'donut' spots. 'Good' probes were accepted as having a mean 'signal to noise ratio' (SNR) value of greater than 5 in more than half of the technical replications.

Data analysis included subtracting the background from median signal intensity for each feature, $\log_2$ transformation and combining technical replicates by taking averages. Subsequently, the signal intensities and flag values of the three biological replicates per isolate were compared and average signal intensities were calculated for only those features that were flagged 'Good' in all the replicates. The values of features that had a 'Bad' flag in either or both the replicates were converted to zero. Finally, PSAW Statistics version 18 (SPSS Inc., IL) was used to examine relationships between the 29 *S. uberis* isolates by constructing a dissimilarity dendrogram using hierarchical cluster analysis with Euclidean distance and between-groups linkage. A Principal Component Analysis (PCA) was also performed using MINITAB® version 16 (Minitab Inc. PA) to identify the probes that revealed maximum difference between the groups (clinical mastitis and LCC isolates).

2.8. Sequence characterization of selected features

Twenty-six probes were selected for sequencing based on PCA analysis (14 features) and probes present mainly in clinical isolates in order to identify and characterise potential virulence determinants (12 features). The probes were amplified in a 50 µL reaction with 2U *Taq* DNA polymerase (Invitrogen, Carlsbad), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of Nested Primers 1 and 2 (Clontech). The cycling conditions were one cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s, and a final extension of 72 °C for 5 min. The PCR products were purified using Qiaquick® PCR Purification Kit and sequenced in both directions by Macrogen Inc. (Seoul, Korea). The sequences were analyzed using Genome Sequence Survey, EST others and Chromosome databases in NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST/). All sequences have been deposited in EMBL Nucleotide Sequence Database (Accession number LN680737 to LN680762).

3. Results

3.1. Cloning of clinical mastitis-specific sequences

Post SSH and amplification of subtracted CM-specific sequences, a library of *S. uberis* tester-specific sequences was obtained following cloning of the secondary PCR products into the pGEM®-T Easy vector. Approximately 363 *E. coli* transformants were obtained from a total of five (W1-W5) independent cloning experiments generating the tester-specific *S. uberis* libraries W1 (Clones W1; 1–41); W2 (W2; Clones 1–117); W3 (W3; Clones 1–73); W4 (W4; Clones 1–76) and W5 (W5; Clones 1–56). Two hundred and seventy two of the recombinants from the libraries W1-W5 were chosen based on their generation of a clear and single band ranging in size from 250 to 1000 bp (Supplementary Fig. 1).

3.2. Subtraction efficiency and validation of the microarray

In order to determine the efficiency of the CM-specific (tester) gDNA subtraction, the gDNA pool of 6 *S. uberis* isolates representing the tester (clinical mastitis) and the other gDNA pool of 6 *S. uberis* isolates representing the driver (low SCC) were hybridized separately on the SDA. Sixty-five (23%) positive spots were observed after hybridizing the driver target with the array. Theoretically, a perfect subtraction should result in removal of all driver-specific gDNA sequences and therefore no signal from any feature when hybridized with driver gDNA. The 65 features that produced signal on hybridization of both driver and tester gDNA were therefore not fully subtracted. Accordingly, the subtraction process was able to isolate tester-specific gDNA sequences with 77% efficiency and the remaining 23% of the features represent non-subtracted sequences.

3.3. Capacity of the SDA to fingerprint S. uberis isolates

Twenty-nine *S. uberis* isolates representing 20 clinical isolates and 9 low SCC isolates were hybridized onto the array to reveal the level of strain differentiation (Table 1). The microarray experiments were carried out according to MIAME guidelines and all data has been deposited in Gene Expression Omnibus (GSE63835). The 29 *S. uberis* isolates tested using the array generated different hybridization patterns allowing discrimination (Fig. 1 and Supplementary Fig. 2). A hierarchical cluster dendrogram was constructed based on the 215 features, after eliminating signal from the 65 un-subtracted features. The 29 isolates grouped into clusters that clearly differentiated between isolates from cows with CM and cows with low SCCs (Fig. 2). Specifically, there were five clusters in the range 7 to 9 distance coefficients. Cluster 1 contained seven isolates of which six were from LCC cows. This clearly indicated that these isolates lack statistically significant linkage with disease status. The second cluster was mixed, consisting of seven isolates; from cows with CM and three from LCC cows, while clusters 3, 4 and 5 contained exclusively CM isolates. Strain (7218 (1)), which clustered at the top of the group LCC isolates hybridized to the least number of probes (21/215), which may indicate a smaller genome size (Baack et al., 2005), while isolate 5838.3, which belongs to the CM group of isolates, hybridized to approximately 50% of the probes (106/215). While some of the clustering observed could be due to sequence differences between the housekeeping that used for MLST typing, most is likely to arise from differences in the remainder of the genome.

3.4. Principal component analysis of hybridization signal from the 29 isolates

In order to identify the DNA fragments that caused the majority of variance in the data (differentiation in between isolates), a principal components analysis (PCA) was performed using hybridization signal from the 29 *S. uberis* isolates. The analysis extracted six different components with significant Eigen values. The first and second principal components explained the majority of the variation (58.4%) of the data (Fig. 3). Therefore, these two dimensions could have most of the variability, in other words, high positive values on the X axis of certain features could explain a large proportion of the total variance found with the full set of features. A PCA analysis using the components 1 and 2 showed that most of the features clustered around zero with only a small number of features forming a loose cluster along the first component axis. Probes that explained the maximum variance hybridized to the DNA from most of the 29 *S. uberis* strains examined. The variance was explained by the difference in signal intensities (median $\log_2$ values) for the particular probe. The most distant features from the cluster and/or closer to zero were then chosen as these features account for most of the variability found across the genotypes, implying that these were the only features that had higher variance and higher mean across the fingerprints. Based on this analysis, only the 14 most distant features from the X axis were chosen for further study (Fig. 3, Table 2). These were the only features that had higher variance and higher mean across the fingerprints; therefore, they could be highly informative.

3.5. Visual identification of isolate-specific features

The PCA alone is not able to detect important polymorphic sequences in the dataset since the methodology only detects features with high variance and high mean signal intensity. Therefore the hybridization patterns (signal) of the 29 isolates were examined visually to detect potentially important features that hybridized mainly to the CM isolates or those that specifically hybridized to a particular group of isolates (Table 2). Twelve group-specific features were identified which were not previously detected by PCA since they had lower means across the fingerprints (Table 2). In addition, a number of isolate-specific probes were identified, for instance, the probe W4-24 was found to be specific for two isolates from cows with clinical mastitis (2988.1 and 3599 (1)).

3.6. Sequencing of selected features

The 14 features chosen through PCA together with 12 strain-specific features identified visually were selected for sequencing. The sequences have been deposited in European Nucleotide Archive (LN680737 to LN680762). A comparative genomic analysis of the 14 probes, selected on the basis of PCA analysis, with genomes of other streptococci using NCBI BLASTN is presented in Table 3. Of particular interest were five features that showed no significant matches in the database, although

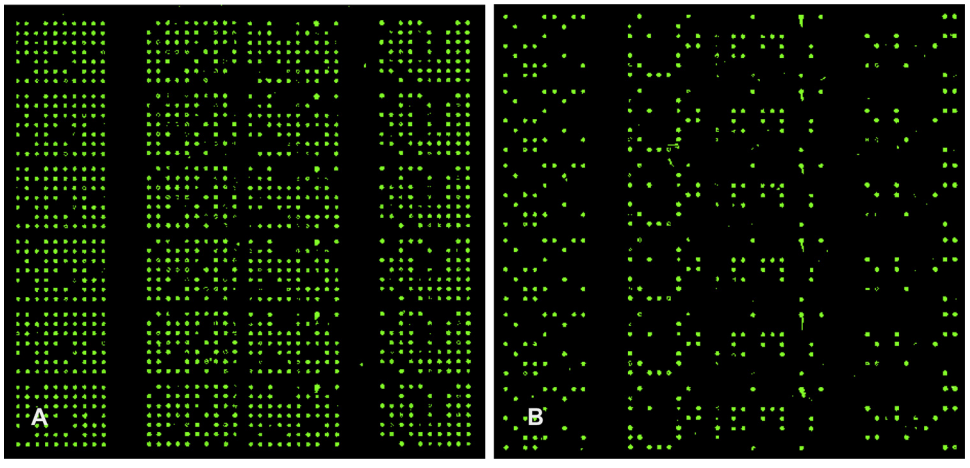

Fig. 1. An example of different hybridization patterns observed for different *S. uberis* isolates. The six sub-arrays representing 6 technical replicates of the 280 features can be seen. Image **(A)** represents isolate from cow with clinical mastitis (5838.3) whereas image **(B)** represents isolate from cow with a low milk somatic cell count (3147.1).

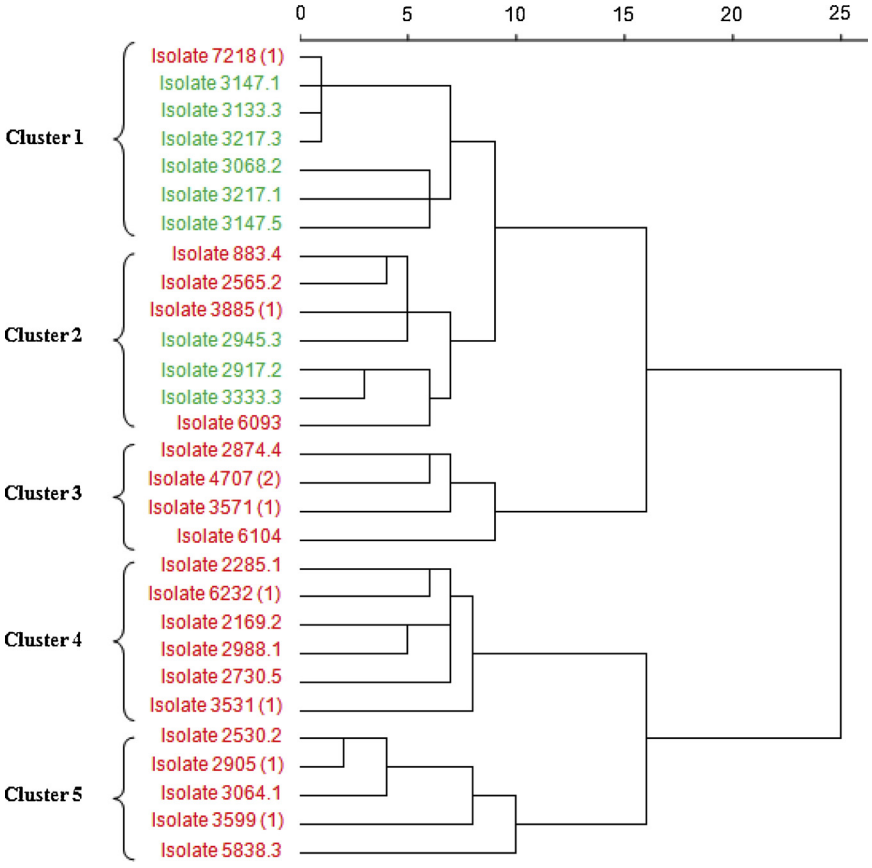

Fig. 2. Dissimilarity dendrogram based on hybridisation pattern for the 29 *S. uberis* isolates: 9 isolates from cows with low somatic cell count (green) and 20 isolates from cows with clinical mastitis (red). The dendrogram was constructed using Squared Euclidean distance and between groups linkage on signal-to-noise ratio of the 215 subtracted fragments. The steps of the dendogram show the combined clusters and the values of the distance coefficients at each step; the values have been rescaled to numbers between 0 and 25, preserving the ratio of the distances between the steps (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

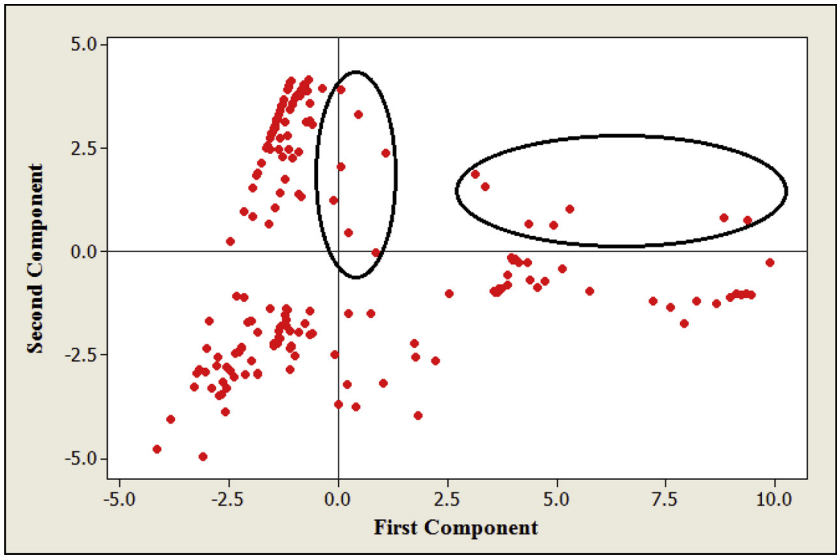

Fig. 3. Principal component analysis (PCA) of the 215 subtracted fragments using signal-to-noise ratio for the 29 *S. uberis* isolates. The first principal component accounts for 34.9% of variation and the second component explained only 23.5% of variation. The ovals indicate features that account for the variability found across the genotypes, i.e only the fourteen most distant features from zero on the X axis were chosen and therefore they are the only features with high variance and a high mean across the fingerprints.

there were clear differences in signal intensity between clinical and non-clinical isolates (Table 4). The remaining nine of the sequenced features did have significant matches in streptococcal genomes. Four probes [W2-1 (LN680738), W4-65 (LN680757), W1-31 (LN680738) and W4-9 (LN680751)] were 98–99% identical to putative microcin immunity protein of *S. uberis* 0104 J genome (Ward et al., 2009). Further, the feature W4-20 (LN680754) was 92% identical to copper importing ATPase of *S. parauberis*. Also, the sequences of the probes W2-16 (LN680740) and W3-73 (LN680749) were 77–78% identical to homologues in *S. parauberis* (fatty oxidation complex protein and 4-hydroxybutyrate CoA-transferase) and *S. iniae* genome (3-hydroxyacyl-CoA dehydrogenase and Glutaryl-CoA dehydrogenase). Additionally, the sequence of probe W1-28 (LN680737) that hybridized to the most *S. uberis* strains tested was 83% identical to membrane protein of *S. macedonicus*. This probe was also 81% identical to sugar transporter of *S. equines* genome while the feature W5-20 (LN680760) was 74% identical to glycosyl transferase of the same organism.

Among the 12 probes identified visually as specifically hybridizing to *S. uberis* CM isolates, six probes did not have any significant matches in the database and may represent novel virulence determinants. Considering the remaining six probes; 50% sequences of W3-30 (LN680746) and W2-38 (LN680743) were 74% identical to putative

Table 2
Streptococcus uberis isolates selected for suppression subtractive hybridization.

Disease status	Isolate ID	PFGE type	Reference
CM	883-4	23	Tomita et al. (2008)
CM	2169-2	11b	Tomita et al. (2008)
CM	2285-1	5a	Tomita et al. (2008)
CM	2530-2	10b	Tomita et al. (2008)
CM	2565-2	29	Tomita et al. (2008)
CM	2730-5	25	Tomita et al. (2008)
CM	2874-4	31a	Tomita et al. (2008)
CM	2988-1	5d	Tomita et al. (2008)
CM	3064-1	12	Tomita et al. (2008)
CM	5838-3	24	Tomita et al. (2008)
CM	6093	34	Tomita et al. (2008)
CM	6104	35	Tomita et al. (2008)
CM	2905(1)	12	Runciman et al. (2010)
CM	3531(1)	26	Runciman et al. (2010)
CM	3571(1)	10	Runciman et al. (2010)
CM	3599(1)	13	Runciman et al. (2010)
CM	3885(1)	45a	Runciman et al. (2010)
CM	4707(2)	35a	Runciman et al. (2010)
CM	6232(1)	16	Runciman et al. (2010)
CM	7218(1)	25	Runciman et al. (2010)
Co	2917-2	L31	Unpublished
Co	2945-3	L35	Unpublished
Co	3068-2	L55	Unpublished
Co	3133-3	17	Tomita et al. (2008)
Co	3147.5	16	Tomita et al. (2008)
Co	3147-1	21	Tomita et al. (2008)
Co	3217-1	15	Tomita et al. (2008)
Co	3217-3	19	Tomita et al. (2008)
Co	3333-3	20	Tomita et al. (2008)

CM, clinical mastitis; Co, low somatic cell count, no clinical or cytological evidence of mastitis, defined as commensal. Isolate identification; suffixes -1, -2, -3, -4 refer to the colony number when several colonies were examined from a single milk sample. All isolates with the exception of 883-4 were isolated from farms in Gippsland Victoria, Australia. Isolate 883-4 was isolates from a farm in the Western district of Victoria, Australia.

transposase gene of *S. salivarius*, with E value of 2e-20 and 6e-20, respectively. Moreover, 43% of the sequence of the probe W5-31 (LN680761) was 95% identical to an integrase of *Facklamia hominis*. The probe W4-24 (LN680755) exhibited 97% protein sequence identity with the putative mannitol-1-phosphate 5-dehydrogenase and sugar phosphotransferase system (PTS), IIA component in the reference *S. uberis* strain 0104 J genome available in the Sanger *S. uberis* database. The probe W4-15 (LN680753) hybridized (77–78%) to a 4-hydroxybutyrate CoA transferase of other streptococcal species. Furthermore, 61% of the sequence of W3-21 (LN680744) was 71% identical to a hypothetical protein of *E. faecalis*. None of the probes identified through PCA or visually hybridised with any of the housekeeping genes used in MLST analysis (*arcC*, *ddl*, *gki*, *recP*, *tdk*, *tpi*, *yqiL*).

4. Discussion

4.1. Validation of clinical mastitis microarray

The subtraction technique used here was able to eliminate about 77% of common DNA sequences between the pool of *S. uberis* isolates from cows with clinical mastitis, and the pool of *S. uberis* isolates from cows with low SCCs (LCC isolates). No study has previously investigated or compared potential virulence-associated genes of *S. uberis* using the SDA technique by generating microarray from pooled subtracted gDNA. The SDA technique used in this study was first developed by Jayasinghe et al. (2007) who performed a very broad subtraction with pooled non-angiosperm gDNA and pooled angiosperm gDNA. This array when validated had 97% subtraction efficiency. Subsequently, a narrower subtraction between pooled Asterids and non-Asterids gDNA (Mantri et al., 2012) resulted in 99% subtraction efficiency. In comparison, a SSH microarray developed by close subtraction between two *Dendrobium* species had only 76% efficiency (Li et al., 2006) which is comparable to the 77% efficiency found in this study, in which 23% non-subtracted features hybridized to both the driver and tester gDNA pool. This may imply that DNA subtraction efficiency is reduced when dealing with groups of isolates within the same species. Nevertheless, the subtraction method was effective in eliminating the majority of the common sequences. It is also likely that some of the subtracted sequences included the housekeeping genes used in the initial MLST analysis to identify suitable isolates for use in driver and tester pools, since most of the clinical isolates belong to GCC 143 or GCC 5 while the low cell count isolates belonged to GCC 86. Another reason for suboptimal subtraction could be the presence of bacteriophage-related genes or other transposable genetic elements in the *S. uberis* isolates. These elements may play a role in the genetic plasticity and substantial recombination observed in the *S. uberis* population (Haenni et al., 2010).

There could also be technical reasons for the failure to totally remove driver sequences by the subtraction. The quantity of the driver pool used might be slightly lower than required. This problem could be overcome if the 30-fold excess of driver which was used in this study according to the manufacturer's instructions, was increased giving a greater excess of driver. This strategy would subtract sequences that are partially homologous between the tester and driver, enriching for those highly tester-specific DNA sequences. Temperature may also play a role in imperfect subtraction. Subtraction hybridization was performed at 68 °C; this is a high temperature and only the highly similar sequences between the driver and tester would be hybridized and subtracted, while for the less similar single-stranded DNA sequences would remain

Table 3
Probes selected after comparing hybridization patterns of the 29 *S. uberis* isolates.

Clone ID*	Significance
W3-72, W1-28, W4-20, W3-57, W4-9, W1-31, W4-65, W3-23, W5-38, W2-16, W2-1, W3-73, W5-20, W2-18	Probes from PCA analysis revealing maximum amount of variation. These probes hybridized with most of the *S. uberis* isolates.
W4-24	Specifically hybridized to clinical isolates (2988.1 and 3599 (1)).
W4-13	Hybridized only to clinical isolates (2730.5, 2874.4, 3531 (1) and 6232 (1)).
W4-5	Hybridized only to clinical isolates (2730.5, 3531 (1), 4707 (2) and 6232 (1)).
W3-21	Specifically hybridized to clinical isolates (2565.2, 2730.5, 3571 (1) and 3599 (1)).
W3-30	Hybridized only to clinical isolates (2169.2, 2285.1, 2874.4, 3531 (1) and 6232 (1)).
W5-31	Specifically hybridized to clinical isolates (2169.2, 2285.1, 3531 (1), 3599 (1) and 6232 (1)).
W4-81	Specifically hybridized to clinical isolates from one farm.
W2-17	Hybridized only to clinical isolates.
W4-80	Hybridized only to clinical isolates.
W4-64	Hybridized only to clinical isolates from two farms
W4-15	Hybridized only to clinical isolates.
W2-38	Hybridized only to clinical isolates.

* Clone identification.

Table 4
Sequence characterization of probes selected after comparing hybridization patterns of the 29 *S. uberis* isolates.

Clone ID[a]	Putative protein	CDS	Strain	Identity	E Value
W2-16[b]	3-hydroxyacyl-CoA dehydrogenase	A0G_0277	NZ_JH930418.1 *S. iniae*	78%	5e-98
	Fatty oxidation complex protein	STP_1025	NC_015558.1 *S. parauberis*	77%	1e-86
W3-73[b]	Glutaryl-CoA dehydrogenase	A0G_0275	NZ_JH930418.1 *S. iniae*	78%	5e-98
	4-hydroxybutyrate CoA-transferase	STP_1026	NC_015558.1 *S. parauberis*	77%	1e-86
W2-1[b]	Microcin immunity protein	SUB0175	NC_012004.1 *S. uberis*	98%	0.0
W4-65[b]	Putative microcin immunity protein	SUB0175	AM946015.1 *S. uberis*	98%	0.0
W5-20[b]	Glycosyl transferase	HMPREF0819_0729	NZ_GL698429.1 *S. equinus*	74%	2e-97
W1-31[b]	Putative microcin immunity protein	SUB0175	AM946015.1 *S. uberis*	99%	0.0
W4-9[b]	Putative microcin immunity protein	SUB0175	AM946015.1 *S. uberis*	99%	0.0
W4-20[b]	Copper importing ATPase	STP_1455	NC_015558.1 *S. parauberis*	92%	0.0
W1-28[b]	Membrane protein	SMA_1309 HMPREF0819_0731	NC_016749.1 *S. macedonicus*	83%	2e-81
	Sugar transporter		NZ_Gl698429.1 *S. equinus*	81%	2e-74
W4-24[c]	Putative mannitol-1-phosphate 5-dehydrogenase	SUB0287 & SUB0288	AM946015.1 *S. uberis*	97%	0.0
	Sugar phosphotransferase system (PTS), IIA component				
W5-31[c]	Integrase	HMPREF9706_00867	43% sequence is identical to NZ_ JH932292.1 *Facklamia hominis*	95%	4e-67
W3-21[c]	Hypothetical protein	EFKG_00175	61% sequence is identical to NZ_GG692670.1 *E. faecalis*	71%	1e-15
W3-30[c]	Putative transposase	SALIVB_0679	50% sequence is identical to NC_015760.1 *S. salivarius*	74%	2e-20
W4-15[c]	4-hydroxybutyrate CoA-transferase	A0G_0276	NZ_JH930418.1 *S. iniae*	78%	5e-98
		STP_1026	NC_015558.1 *S. parauberis*	77%	1e-86
W2-38[c]	Putative transposase	SALIVB_0679	50% sequence is identical to NC_015760.1 *S. salivarius*	74%	6e-20

[d]NA, not applicable. The other 11 isolates showed no significant matches.
[a] Clone identification.
[b] Hybridized to most of the *S. uberis* isolates from PCA.
[c] Specifically hybridized to certain clinical isolates.

Table 5
Log_{10} signal intensity of probes identified by principal component analysis.

Clone ID	EMBL Sequence ID	Signal intensity of	
		Clinical isolates	Commensals
W3-72	LN680748	10.13	0
W3-57	LN680747	10.19	0
W3-23	LN680745	9.58	2.71
W5-38	LN680762	10.17	2.96
W2-18	LN680742	12.79	10.4

unhybridized to be amplified by PCR, and printed on the array. Therefore, reducing the temperature of the two rounds of subtraction hybridization may improve the SDA technique and elevate the percentage of the sequences that are partially homologous between the pools to be subtracted (Gadgil et al., 2002).

4.2. Clustering of clinical and low SCC S. uberis

The ability of this SDA technology to differentiate clearly between isolates from cows with CM (Fig. 2, clusters 3, 4 5) and cows with no clinical, microbiological or cytological evidence of mastitis (LCC isolates) (Fig. 2, cluster 1) indicates that most cases of clinical mastitis are caused by a subset of *S. uberis* strains that differ in genetic attributes from most other strains. Other investigators using MLST (Tomita et al., 2008), experimental challenge, (Tassi et al., 2013), microarray (Lang et al., 2009), and PFGE (Abureema et al., 2014) have also concluded that specific strains of *S. uberis* are adapted to for transmission between cows and ability to cause mastitis, while others behave predominantly as environmental strains. A strain's complement of virulence-associated factors therefore appears to be a significant determinant of pathogenicity; but other factors, including teat end health, breed and age also determine whether an individual animal develops clinical mastitis] (Lacy-Hulbert and Hillerton, 1995), explaining the mixture of clinical and non-clinical isolates appearing in Cluster 2 (Fig. 2). While some of this clustering could be attributed to un-subtracted sequences due to differences in housekeeping genes between the two gene pools, these differences are likely to contribute to only a small part of the differences between the strains shown in Fig. 2.

4.3. Sequencing of selected probes

Sequences identified by PCA analysis as prevalent in all isolates.

All the 14 probes identified by PCA hybridized to most of the clinical and low SCC isolates of *S. uberis* and nine of their sequences had significant matches within the streptococcal genomes (Table 3). In agreement with other studies showing that genes related to metabolism are numerous in the *S. uberis* core genome (Lang et al., 2009; Ward et al., 2009; Hossain et al., 2015), the predicted proteins of five probes [W1-28 (LN680737), W2-16 (LN680740), W3-73 (LN680749), W4-20 (LN680754), W5-20 (LN680760)] exhibited extensive protein sequence identity with proteins involved in carbohydrate and lipid metabolism. Four probes with sequence homology to genes encoding microcin immunity proteins were also identified. Microcins and their corresponding immunity proteins are common in the genomes of *S. uberis* (Ward et al., 2009). As their function is to kill closely related bacterial species competing for the same environmental niches (bovine skin, tonsils, gastrointestinal tract), it is not surprising that the presence of these four probes did not differ between clinical and non-clinical isolates. The five probes that showed no significant matches in the database were of particular interest, since clinical isolates displayed more intense signals than non-clinical isolates (Table 4). This suggests that these probes may represent *S. uberis*-specific factors involved in establishment of infection in the bovine mammary gland.

4.4. Sequences found more frequently in clinical isolates than in isolates from cows with low SCCs

We suggest that the six probes that were highly associated with *S. uberis* isolates, and had no matches in the database, may represent novel virulence genes. The remaining sequences specific to CM isolates were associated with metabolic pathways (2 probes), mobile genetic elements (3 probes) and hypothetical proteins (7 probes). Other studies using SSH followed by dot blot analysis (Tomita, PhD thesis, RMIT University, 2008) and WGS (Hossain et al., 2015) also identified

sequences in clinical *S. uberis* that encoded genes related to carbohydrate metabolism. The corresponding gene products may contribute significantly to growth or survival of *S. uberis* in the bovine mammary gland since *S. uberis* grows predominantly in ductular, tissue and the lumen of secretory alveoli and rarely invades udder tissue (Thomas et al., 1994). Supporting a significant role of genes involved in metabolism in the establishment of mastitis, Taylor et al. (2003) found that several genes, including those encoding proteins involved in sugar metabolism, were upregulated during growth in milk *in vitro*. The authors suggested that these metabolic genes might contribute to establishment of mastitis, enabling growth in milk by facilitating the uptake of sugars and peptides.

The identification of transposable elements, including bacteriophages and transposons, specific to isolates from cows with CM, in the present study as well as a recent study in our laboratory (Tomita, PhD thesis, RMIT University Australia, 2008) suggests that these elements provide some advantage to the organism, possibly encoding factors for establishment of infection in the bovine mammary gland. Our results extend earlier studies that identified bacteriophage-derived sequences and laterally-acquired elements in the variable region of the genome of *S. uberis* O140 J, isolated from a clinical case of bovine mastitis in 1972 (Lang et al., 2009; Ward et al., 2009).

Another sequence of interest, W3-21 (LN680744), was analogous to a hypothetical sequence present in *E. faecalis* and other Gram positive bacteria. The corresponding gene product might facilitate initiation of infection through adherence to damaged tissue by binding to fibronectin and other extracellular matrix proteins, as proposed for *E. faecalis* by Rozdzinski et al. (2001).

Our findings are broadly in line with those of a recent study (Hossain et al., 2015) that examined sequences of 13 *S. uberis* isolates from cases of clinical and sub-clinical mastitis, including one isolate from a cow with clinical mastitis that was later shown to be non-virulent in a lactating cow mastitis model. Several virulence genes were identified in most of the genomes, but genome content did not distinguish between the groups. One possible reason for this could be that no isolates from cows with low milk cell counts were included in the study. Other reasons, as suggested by the authors, could be the complex dynamic relationship that exists between, *S. uberis*, its, host and environment.

5. Conclusion

We developed an SDA array that had high discriminatory power and the capacity to differentiate between isolates of *S. uberis* causing clinical mastitis from those not associated with mastitis. Gene sequences potentially representing novel virulence-associated genes, sequences associated with carbohydrate and lipid metabolism, and certain mobile genetic elements were enriched in clinical isolates and may enhance their capacity to grow in milk and invade the bovine udder. The methodology described here has the potential to identify groups of virulence-related genes and to characterize gene products that could in future be the basis for a successful mastitis vaccine. The methodology could also be used for *S. uberis* strain and virulence typing, since each strain appears to have a characteristic distribution of putative virulence-associated sequences. This information could potentially be used to alert farmers of strains within a herd that may fail to respond to treatment, become persistent or have greater potential for transmission between cows.

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Declaration of Competing Interest

None.

References

Abureema, S.F., Smooker, P., Malmo, J., Deighton, M.A., 2014. Molecular epidemiology of recurrent clinical mastitis due to *Streptococcus uberis*: evidence of both an environmental source and of recurring infection with the same strain. J. Dairy Sci. 97, 85–290.

Almeida, R.A., Luther, D.A., Park, H.-M., Oliver, S.P., 2006. Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM). Vet. Microbiol. 115, 183–191.

Baack, E.J., Whitney, K.D., Rieseberg, L.H., 2005. Hybridization and genome size evolution: timing and magnitude of nuclear DNA content increases in Helianthus homoploid hybrid species. New Phytol. 167, 623–630.

Botrel, M.A., Haenni, M., Morignat, E., Sulpice, P., Madec, J.Y., Calavas, D., 2010. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone-Aples, France. Foodborne Pathog. Dis. 7, 479–487.

Bradley, A.J., Leach, K.A., Breen, J.E., Green, L.E., Green, M.J., 2007. Survey of the incidence and etiology of mastitis on dairy farms in England and Wales. Vet. Rec. 160, 253–258.

Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D., 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. PNAS 93, 6025–6030.

Egan, S.A., Ward, P.N., Watson, M.T., Field, R.E., Leigh, J.A., 2012. Vru (sub0144) controls expression of proven and putative virulence determinants and alters the ability of *Streptococcus uberis* to cause disease in dairy cattle. Microbiology 158, 1581–1592.

Field, T.R., Ward, P.N., Pedersen, L.H., Leigh, J.A., 2003. The hyaluronic acid capsule of *Streptococcus uberis* is not required for the development of infection and clinical mastitis. Infect. Immun. 71, 132–139.

Gadgil, C., Rink, A., Beattie, C., Hu, W.S., 2002. A mathematical model for suppression subtractive hybridization. Comp. Funct. Genom. 3, 405–422.

Hacker, J., Blum-Oehler, G., Muhldorfer, I., Tschape, H., 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Molec. Microbiol. 23, 1089–1097.

Haenni, M., Saras, E., Bertin, S., Leblond, P., Madec, J.Y., Payot, S., 2010. Diversity and mobility of integrative and conjugative elements in bovine isolates of *Streptococcus agalactiae, S. dysgalactiae* subsp. dysgalactiae, and *S. uberis*. Appl. Environ. Microbiol. 76, 7957–7965.

Hossain, M., Egan, S.A., Coffey, T., Ward, P.N., Wilson, R., Leigh, J.A., Emes, R.D., 2015. Virulence-related sequences; insights provided by comparative genomics of *Streptococcus uberis* of differing virulence. BMC Genom. 16, 334–346.

Jayasinghe, R., Kong, S., Coram, T.E., Kaganovitch, J., Xue, C.C., Li, C.G., Pang, E.C., 2007. Construction and validation of a prototype microarray for efficient and high-throughput genotyping of angiosperms. Plant Biotechnol. J. 5, 282–289.

Lacy-Hulbert, S.J., Hillerton, J.E., 1995. Physical characteristics of the bovine treat canal and their influence on the susceptibility to streptococcal infection. J. Dairy Res. 62, 395–404.

Lang, P., Lefebure, T., Wang, W., Zadoks, R.N., Schukken, Y., Stanhope, M.J., 2009. Gene content differences across strains of Streptococcus uberis identified using oligonucleotide microarray comparative genomic hybridization. Infect. Genet. Evol. 9, 179–188.

Leigh, J.A., Egan, S.A., Ward, P.N., Field, T.R., Coffey, T.J., 2010. Sortase anchored proteins of *Streptococcus uberis* play major roles in the pathogenesis of bovine mastitis in dairy cattle. Vet. Res. 41, 63–79.

Leigh, J.A., Lincoln, R.A., 1997. *Streptococcus uberis* acquires plasmin activity following growth in the presence of bovine plasminogen through the action of its specific plasminogen activator. FEMS Microbiol. Lett. 154, 123–129.

Li, T.X., Wang, J.K., Bai, Y.F., Lu, Z.H., 2006. Diversity suppression-subtractive hybridization array for profiling genomic DNA polymorphisms. J. Integr. Plant Biol. 48, 460–467.

Mantri, N., Olarte, A., Li, C.G., Xue, C., Pang, E.C.K., 2012. Fingerprinting the Asterid Species using subtracted diversity array reveals novel species-specific sequences. PLoS One 7, e34873.

Olarte, A., Mantri, N., Nugent, G., Li, C.G., Xue, C., Pang, E.C.K., 2013a. A gDNA microarray for genotyping Salvia species. Mol. Biotechnol. 54, 770–783.

Olarte, A., Mantri, N., Nugent, G., Pang, E.C.K., 2013b. Subtracted diversity array identifies novel molecular markers including retrotransposons for fingerprinting Echinacea species. PLoS One 8, e70347.

Olde Reikerink, R.G., Barkema, H.W., Kelton, D.F., Scholl, D.T., 2008. Incidence rate of clinical mastitis on Canadian dairy farms. J. Dairy Sci. 91, 1366–1377.

Rozdzinski, E., Marre, R., Susa, M., Wirth, R., Muscholl-Silberhorn, A., 2001. Aggregation substance-mediated adherence of Enterococcus faecalis to immobilized extracellular matrix proteins. Microbial. Pathog. 30, 211–220.

Runciman, D.J., Malmo, J., Deighton, M., 2010. The use of an internal teat sealant in combination with cloxacillin dry cow therapy for the prevention of clinical and subclinical mastitis in seasonal calving dairy cows. J. Dairy Sci. 93, 4582–4591.

Shum, L.W., McConnel, C.S., Gunn, A.A., House, J.K., 2009. Environmental mastitis in high-producing dairy herds in New South Wales. Aust. Vet. J. 87, 469–475.

Smith, A.J., Kitt, A.J., Ward, P.N., Leigh, J.A., 2002. Isolation and characterization of a mutant strain of *Streptococcus uberis*, which fails to utilize a plasmin derived beta-casein peptide for the acquisition of methionine. J. Appl. Microbiol. 93, 631–639.

Smith, A.J., Ward, P.N., Field, T.R., Jones, C.L., Lincoln, R.A., Leigh, J.A., 2003. MtuA, a lipoprotein receptor antigen from Streptococcus uberis, is responsible for acquisition of manganese during growth in milk and is essential for infection of the lactating bovine mammary gland. Infect. Immun. 71, 4842–4849.

Tassi, R., McNeilly, T.N., Fitzpatrick, J.L., Fontaine, M.C., Reddick, D., Ramage, C., Lutton, M., Schukken, Y.H., Zadoks, R.N., 2013. Strain-specific pathogenicity of putative host-adapted and nonadapted strains of *Streptococcus uberis* in dairy cattle. J. Dairy Sci. 51, 29–45.

Taylor, D.L., Ward, P.N., Rapier, C.D., Leigh, J.A., Bowler, L.D., 2003. Identification of a differentially expressed oligopeptide binding protein (OppA2) in *Streptococcus uberis* by representational difference analysis of cDNA. J. Bacteriol. 185, 5210–5219.

Thomas, L.H., Haider, W., Hill, A.W., Cook, R.S., 1994. Pathologic findings of experimentally induced *Streptococcus uberis* infection in the mammary gland of cows. Am. J. Vet. Res. 55, 1723–1728.

Tomita, T., 2008. Subspecies Distribution and Virulence Factors of *Streptococcus Uberis*. PhD Thesis. RMIT University, Melbourne Australia.

Tomita, T., Meehan, B., Wongkattiya, N., Malmo, J., Pullinger, G., Leigh, J., Deighton, M., 2008. Identification of *Streptococcus uberis* multilocus sequence types highly associated with mastitis. Appl. Environ. Microbiol. 74, 114–124.

Ward, P.N., Holden, M.T.G., Leigh, J.A., Lennard, N., Bignell, A., Barron, A., Clark, L., Quail, M.A., Woodward, J., Barrell, B.G., Egan, S.A., Field, T.R., Maskell, D., Kehoe, M., Dowson, C.G., Chanter, Nl., Whatmore, A.M., Bentley, S.D., Parkhill, J., 2009. Evidence for niche adaption in genome of the bovine pathogen *Streptococcus uberis*. BMC Genom. 10, 54–71.