Transcriptome profiling of *Streptococcus uberis*-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model

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ABSTRACT

Streptococcus uberis is a prevalent causative organism of mastitis and resides naturally in the environment of the dairy cow making prevention of the disease difficult. A bovine cDNA microarray comprising approximately 22,000 expressed sequence tags was used to evaluate the transcriptional changes that occur in the mammary gland after the onset of clinical *Strep. uberis* mastitis. Five lactating Friesian heifers were intramammary infused in an uninfected quarter with approximately 1,000 to 1,500 cfu of a wild-type strain of *Strep. uberis*. Microarray results showed that *Strep. uberis* mastitis led to the differential expression of more than 2,200 genes by greater than 1.5-fold compared with noninfected control quarters. The most highly upregulated genes were associated with the immune response, programmed cell death, and oxidative stress. Quantitative real-time reverse transcription PCR analysis confirmed the increase in mRNA expression of immune-related genes complement component 3, clusterin, IL-8, calgranulin C, IFN- γ , IL-10, IL-1 β , IL-6, toll-like receptor-2, tumor necrosis factor- α , serum amyloid A3, lactoferrin, LPS-binding protein, and oxidative stress-related genes metallothionein 1A and superoxide dismutase 2. In contrast, a decrease of mRNA levels was observed for the major milk protein genes. Bovine mammary epithelial cells in culture challenged with the same *Strep. uberis* strain used to induce clinical mastitis in the in vivo animal experiment did not cause a change in the mRNA levels of the immune-related genes. This suggests that the expression of immune-related genes by mammary epithelial cells may be initiated by host factors and not *Strep. uberis*. However, challenging epithelial cells with different *Strep. uberis* strains and *Staphylococcus aureus* resulted in an increase in the mRNA expression of a subset of the immune-related genes measured.

In comparison, an *Escherichia coli* challenge caused an increase in the majority of immune-related genes measured. Results demonstrate the complexity of the bovine mammary gland immune response to an infecting pathogen and indicate that a coordinated response exists between the resident, recruited, and inducible immune factors.

Key words: mastitis, bovine, microarray, *Streptococcus uberis*

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland and is usually a consequence of microbial infection (Jain, 1979). It is a highly prevalent and costly disease for the dairy industry worldwide (Blosser, 1979; DeGraves and Fetrow, 1993). *Streptococcus uberis* is a major causative agent of IMI in dairy cows both in countries with pasture-based management systems and seasonal calving (Hillerton et al., 1993; McDougall, 2002) and where cattle are housed and have year-round calving (Hillerton et al., 1993; McDougall, 2002). *Streptococcus uberis* resides naturally in the environment and has been isolated from the soil, races, and feces, in addition to various anatomical regions in the cow (Cullen, 1966; Khan et al., 2003; Lacy-Hulbert et al., 2005). Currently, it is not effectively controlled using existing control methods (Leigh, 1999), with the primary means being through administration of antibiotics after the identification of infection. However, with ongoing international pressure to decrease antibiotic use in agriculture due to its perceived link to increasing antibiotic resistance (Teuber, 2001; Phillips et al., 2004), an understanding of the bovine immune response to mastitis may elucidate alternate avenues of combating this disease.

The immune response associated with mastitis is a very complex biological process and involves resident, recruited, and inducible immune factors (Rainard and Riollet, 2006). The innate immune response is the predominant defense during the early stages of infection and is induced rapidly at the site of infection (Sordillo

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et al., 1997). Distinctive differences in the innate immune response have been reported after the infusion of gram-positive and gram-negative organisms into the mammary gland (Bannerman et al., 2004a,b). Although the systemic and innate immune response for gram-negative organisms appears similar, differences were evident in these responses for 2 prolific mastitis-causing gram-positive pathogens, *Staphylococcus aureus* and *Strep. uberis* (Bannerman et al., 2004a,b). These studies highlight the variability of the innate immune response to different pathogens and establish the importance of understanding the mediators involved in the recognition and signaling of invasion by specific pathogens. Understanding of the immune response of the bovine mammary gland during *Strep. uberis* mastitis is still very rudimentary. However, this broad-spectrum response offers potential avenues of research for increasing the control and prevention of mastitis through the numerous immune and epithelial cell-derived molecules, which respond to the presence of pathogens in the mammary gland (Paape et al., 2000; Bannerman et al., 2004a).

Microarray technology enables the examination of complex interactions between the host and bacterial pathogens (Zheng et al., 2006). It has become a valuable tool in experimental biology, because it enables the simultaneous generation of quantitative data relating to the differential expression of thousands of genes (Schna et al., 1995; Shalon et al., 1996). The identification of genes whose expression is altered in response to bacterial pathogens and the ability to classify these into biologically functional groups aid in the overall understanding of the host response to a specific pathogen (Zheng et al., 2006). The purpose of this study was to analyze the changes in gene expression in vivo in the bovine mammary gland in response to *Strep. uberis* and to compare this to other significant mastitis pathogens in vitro to identify the specific role of the epithelial cells using a bovine mammary epithelial cell (**bMEC**) model. The use of microarray technology allowed a comprehensive investigation into the complex transcriptional changes that occur after the onset of clinical *Strep. uberis* mastitis and subsequent comparative in vitro studies of *Strep. uberis* and other mastitis pathogens, highlighted differences in the immune response to these pathogens, and improves the overall understanding of this disease.

MATERIALS AND METHODS

Animals and Sample Collection

A healthy front and rear quarter of the mammary gland of each of 5 Friesian heifers (mid-late lactation)

was infused with approximately 1,000 to 1,500 cfu of a wild-type strain of *Strep. uberis* (*Strep. uberis* 233, New Zealand clinical mastitis isolate, nonencapsulated) in 1 mL of quarter-strength Ringer's solution (Oxoid, Hampshire, UK) immediately after an afternoon milking (Milner et al., 1997). One of these quarters was selected for analysis (Table 1). The strain of *Strep. uberis* was in the log growth phase, and serial dilution plate counts were performed to enumerate the inoculum before infusion. The *Strep. uberis* strain used had been previously isolated from a cow with clinical mastitis. The cows were observed for visual signs of clinical mastitis. Animals had no previous episodes of clinical or subclinical mastitis. Quarter milk samples on the day of trial confirmed that the sampled quarters were free from mastitis pathogens and had less than 150,000 cells/mL. The animals were slaughtered at the Ruakura abattoir (Hamilton, New Zealand) using standard commercial procedures (electrical stunning followed by exsanguination). Mammary alveolar tissue (approximately 30 g) was collected from the middle of the upper one-third of one of the *Strep. uberis*-infused quarters displaying clinical signs of mastitis, specifically clots in the milk, and a control quarter that had the lowest SCC and negative bacteriology data (Table 1). Samples were snap-frozen in liquid nitrogen for subsequent RNA and protein extraction. All procedures were approved by the Ruakura Animal Ethics Committee in accordance with the 1999 Animal Welfare Act of New Zealand. Milk samples were taken for bacteriological examination and SCC to determine infection status 1 h before tissue sampling (Stelwagen and Lacy-Hulbert, 1996).

Array Preparation

Bovine cDNA libraries were generated at AgResearch from a variety of tissues from both dairy and beef breeds as described by Singh et al. (2008). Expressed sequence tags (**EST**) were amplified, and a microarray consisting of 21,378 spots was printed onto poly-L-lysine-coated glass slides using an ESI array robot (ESI, Toronto, Canada) at the University of Otago Genomics Facility (Dunedin, New Zealand) as described by Baird et al. (2004).

Target Preparation and Hybridization

Labeled cDNA was prepared as described in Singh et al. (2008). Briefly, total RNA from mammary alveolar tissue was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and purified using an RNeasy kit (Qiagen, Valencia, CA). Ribonucleic acid integrity

Table 1. Somatic cell count and bacteriology results for individual quarters

Cow	Infection status	Quarter sampled ¹	SCC (10 ³ cells/mL)	Time taken for clinical signs (h)	Bacteriology comment
1	Noninfected	LF	38		No specific growth
	Clinical infection	RB	16,147	60	Heavy growth of <i>Streptococcus uberis</i>
2	Noninfected	LB	30		No specific growth
	Clinical infection	RF	2,909	60	Heavy growth of <i>S. uberis</i>
3	Noninfected	RF	219		No specific growth
	Clinical infection	RB	13,865	132	Heavy growth of <i>S. uberis</i>
4	Noninfected	LB	116		No specific growth
	Clinical infection	RB	18,912	132	Heavy growth of <i>S. uberis</i>
5	Noninfected	RB	93		No specific growth
	Clinical infection	LF	17,297	60	Heavy growth of <i>S. uberis</i>

¹LF = left front; LB = left back; RF = right front; RB = right back.

was confirmed by denaturing agarose gel electrophoresis and quantified by UV spectrophotometry. Labeled cDNA was produced from 25 µg of total RNA by oligo (dT)-primed reverse transcription and incorporation of aminoallyl-deoxyuridine triphosphate, using Moloney murine leukemia virus reverse transcriptase (Ambion Inc., Austin, TX). The amino-modified cDNA was coupled to reactive forms of Cy3 and Cy5 dyes using the Ambion amino allyl cDNA labeling kit (Ambion Inc.). The slides were hybridized with the labeled cDNA using ULTRAhyb (Ambion Inc.) as described previously (Baird et al., 2004). A dye swap was incorporated into the experimental design to eliminate bias related to the incorporation of the 2 different dyes. The microarray data were read using a GenePix 4200a Scanner (Axon Instruments Inc., Union City, CA), and the images were analyzed using GenePix Pro 3.0 software (Axon Instruments Inc.), which included automated and manual flagging of bad spots.

Microarray Normalization and Analysis

The microarray expression data for each slide were normalized following the procedure of Baird et al. (2004) using GenStat (VSN International, Hemel Hempstead, UK). To determine which EST were differentially expressed, the normalized data for each EST from all 10 slides were averaged, correcting for dye reversal, and the *P*-value was reported from a *t*-test. Expressed sequence tags were excluded from further analysis if they had more than 5/10 bad spots. A total of 19,760 EST were selected for further analysis representing 10,337 distinct contiguous sequences (**contig**), of which 6,812 were represented only once. Of the selected EST, 16,409 matched to entries in the human RefSeq database, and these represented 8,216 different contig. The 3,351 EST with no RefSeq matches represented 2,121 different contig. These remaining EST were sorted based on the mean normalized (or corrected) log ratio.

Microarray Data Interpretation

To annotate the microarrays, all the EST sequences, including those publicly deposited in National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), were assembled into contig using the DNA assembly program CAP3 (Huang and Madan, 1999) after an initial clustering step using BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>; Altschul et al., 1990). The corresponding human RefSeq (RefSeq release as at September 15, 2006) for each contig represented on the microarray array was obtained using BLAST ($E < 1 \times 10^{-10}$). In cases in which the contig matched more than 1 transcript variant of a gene, the top hit was used. Expressed sequence tags were considered differentially expressed if they were up- or down-regulated by ≥ 1.5 -fold or ≤ -1.5 -fold, respectively ($P < 0.05$), based on their *C_logRatio*. Contigs represented by multiple EST were considered as differentially expressed if at least one-third of the EST were up- or downregulated by ≥ 1.5 -fold ($P < 0.05$) and if the average of the *C_logRatio* for all EST equated to differential expression of ≥ 1.5 -fold. Ontological classification and further annotation of the genes encoded by the differentially expressed EST were performed using the Database for Annotation, Visualization and Integrated Discovery (**DAVID**; Dennis et al., 2003). The human RefSeq representing differentially expressed EST were submitted to DAVID with a background list including all human RefSeq ($E < 1 \times 10^{-10}$) on the microarray ($P < 0.05$). The Fisher's exact *P*-value was used to measure the gene enrichment and identifies EST that are specifically regulated after the onset of *Strep. uberis* infection. Statistically significant gene ontology terms ($P < 0.05$) are reported.

Quantitative Real-Time Reverse Transcription PCR Analysis

First-strand cDNA was generated from RNA samples using the SuperScript III Reverse Transcriptase First-

Strand Synthesis System kit (Invitrogen; Singh et al., 2008). Before use as templates in PCR amplification, cDNA samples were diluted 5-fold in 10 mM Tris pH 8.0. The cDNA samples were assayed on 96-well optical plates (Applied Biosystems, Foster City, CA) in duplicate using an ABI PRISM 7700 System (Applied Biosystems). Each plate included an assay for an endogenous control gene (ubiquitin) as well as primers for the target gene of interest. Primer sequences for complement component 3 (**C3**), clusterin, cluster of differentiation (**CD**) 55 molecule (decay accelerating factor for complement, **DAF**), chemokine (C-X-C motif) ligand 1 (**CXCL1**), chemokine (C-X-C motif) ligand 2 (**CXCL2**), IL-1 β , IL-6, IL-8, IL-10, calgranulin C (**S100A12**), IFN- γ , LPS-binding protein (**LBP**), serum amyloid A3 (**SAA3**), transforming growth factor- β , toll-like receptor 2 (**TLR2**), and tumor necrosis factor- α (**TNF- α**) were designed from published nucleic acid sequences using Primer 3 software (Rozen and Skaletsky, 2000) and are described in Supplementary Table 1 (<http://jds.fass.org/content/vol92/issue1/>). Primer sequences for α -LA, β -LG, α S1-CN, β -CN, κ -CN, lactoferrin, lingual antimicrobial peptide (**LAP**), superoxide dismutase 2 (**SOD2**), metallothionein 1A, and ubiquitin are described previously (Singh et al., 2008). Each quantitative reaction contained 7.5 μ L of SYBR Green Master Mix (Applied Biosystems), 1 μ L of cDNA template, and 300 nM of each primer pair. An initial denaturation step of 95°C for 10 min preceded 40 cycles of 95°C for 15 s, 56 to 62°C for 30 s, 72°C for 30 s, and 78°C for 10 s. A dissociation curve of the amplified products was acquired after the 40 cycles by a further run of 95°C for 15 s, then 60°C for 15 s followed by a slow ramp up to 95°C for 15 s. The PCR products were verified by sequencing (Waikato DNA Sequencing Facility, Hamilton, New Zealand). Relative quantification of gene expression was performed using the standard curve method (Applied Biosystems). Briefly, standard curves of serial dilutions of PCR products generated from each primer pair were used to calculate the relative amounts of each gene in the cDNA samples. The amount of target gene in each sample was normalized to the endogenous control gene (ubiquitin) and the resulting values were base-10 logarithm-transformed. The levels of mRNA were then backtransformed and expressed as the mean fold change $\pm$ SEM relative to the noninfected mean. Differences between the infected and noninfected means were analyzed by ANOVA of the log-transformed data in GenStat, with the LSD between means calculated ($P < 0.1$, $P < 0.05$, $P < 0.01$, $P < 0.001$).

Cell Culture

Streptococcus uberis 233 and 262 (New Zealand clinical mastitis isolate, encapsulated), *Escherichia coli* (ATCC25922), and *Staph. aureus* (ATCC25923) were collected by centrifugation ($10,000 \times g$, 30 min) after overnight growth in Todd Hewitt broth, Luria-Bertani broth, and tryptic soy broth, respectively. Bacterial concentrations were defined by plating serial dilutions. The bacterial cells were washed in PBS, resuspended at a concentration of 1×10^{10} cfu/mL in $1 \times$ PBS, 10% glycerol, and stored at -80°C . Before use, bacterial cells were heat-killed at 65°C for 30 min, and inactivation efficiency was verified on blood agar plates. Cells were resuspended at the required concentration in cell culture media. Plating of dilution series was used to verify cell counts.

Primary cultures of bMEC were isolated from a slaughtered third trimester pregnant cow. Approximately 100 g of alveolar tissue was removed immediately after slaughter and transported back to the laboratory in Hanks' balanced salt solution (**HBSS**) containing antibiotic-antimycotic (Invitrogen). Tissue samples were rinsed several times in HBSS and were then cut into 1-cm² pieces, removing fat and connective tissue. These pieces were then injected with digest mixture [HBSS with 0.4% BSA (wt/vol), 2 mM glutamine, MEM AA solution (Invitrogen), 5.5 mM glucose, 1.2 mg/mL of collagenase IV (Worthington Biochemical Corp., Lakewood, NJ), 0.02 mg/mL of DNase I, 0.5 mg/mL of hyaluronidase (Sigma, St. Louis, MO), 1 μ g/mL of cortisol, 5 μ g/mL of insulin, and antibiotic-antimycotic (Invitrogen)]. Approximately 50 g of tissue was placed into 500-mL conical flasks containing 250 mL of digest mixture, and digestion proceeded for 2 h in an orbital shaker (37°C ; 110 rpm). After the digestion period, cellular debris and single cells were removed by centrifugation ($80 \times g$; 5 min). The remaining tissue samples were minced with opposing scalpel blades and digested for a further 3 h. Cells were then filtered through 150- μ m mesh and centrifuged ($100 \times g$; 10 min). Cells were washed in HBSS before fractionation on a Percoll (Sigma) gradient. Epithelial cells banded at 1.03 to 1.05g/mL fractions after centrifugation at $800 \times g$ for 20 min. The resulting cells were washed in HBSS and cryopreserved at a concentration of 20×10^6 cells/mL in 90% fetal calf serum, 10% dimethyl sulfoxide.

Primary cell aliquots were thawed and grown to confluence in 10-cm² plates in a composite Medium 199/Hams F12 medium (Invitrogen) supplemented with 45 mM sodium bicarbonate, 0.4 mM sodium acetate, 10

mM HEPES, 5% fetal calf serum, 5% horse serum, 5 μ g/mL of insulin, and 1 μ g/mL of cortisol. Cells were then trypsinized and split into two 6-well plates. The cells were cultured until confluence before subsequent addition of fresh media with or without (control) heat-killed bacteria at final concentrations of 1×10^9 cfu/mL, representing a multiplicity of infection of approximately 1,000. Each treatment was performed in triplicate, and the experiment was repeated. After incubation for 12 h, the cells were washed with PBS and harvested by trypsinization using trypLE (Invitrogen) for 10 min. Total RNA was extracted from the cells and cDNA generated for use in quantitative real-time reverse transcription PCR (qRT-PCR) as described above. The differences between means and fold changes relative to the control were analyzed as described above.

RESULTS

The microarray analysis examined gene expression changes in the alveolar mammary tissue from quarters showing clinical signs of mastitis induced by infusion with *Strep. uberis* compared with healthy noninfected quarters from the same cows. A significance threshold of $P < 0.05$, with at least 1.5-fold change, was used in all comparisons. There were 1,083 different genes upregulated (including 326 with no human RefSeq: GenBank accession number) and 1,212 genes downregulated (including 239 with no human RefSeq: GenBank accession number) in *Strep. uberis*-infected mammary alveolar tissue compared with the noninfected tissue. Of these genes, there were 15 that were upregulated and only 1 that was downregulated by >5-fold in the infected tissue compared with the noninfected tissue (Supplementary Tables 2 and 3; <http://jds.fass.org/content/vol92/issue1/>). These highly upregulated genes include the antiapoptotic SON DNA-binding protein (7.26-fold); the calcium-binding protein, S100A12 (7.26-fold), which is involved in the inflammatory response; and the acute-phase protein, SAA3 (6.91-fold). The complete lists of differentially expressed genes ($P < 0.05$, with at least a 1.5-fold change) with human RefSeq ($E < 1 \times 10^{-10}$) and DAVID classifications are provided in Supplementary Tables 2 and 3 (<http://jds.fass.org/content/vol92/issue1/>).

The differentially expressed genes had many different DAVID classifications. The upregulated genes with the greatest fold changes had functions predominantly associated with a response to the presence of *Strep. uberis*, such as response to biotic stimulus, defense response, immune response, response to stimulus, and response to pest, pathogen, or parasite. Genes involved in homeostasis were also significantly upregulated in the *Strep. uberis*-infected samples compared with non-

infected samples (Table 2; Supplementary Table 2; <http://jds.fass.org/content/vol92/issue1/>). In contrast, the downregulated genes had functions pertaining to metabolism, development, and DNA damage (Table 3; Supplementary Table 3; <http://jds.fass.org/content/vol92/issue1/>).

Genes with functions related to the response to *Strep. uberis* that were upregulated were more specifically classified to reactive oxygen species, acute-phase response, inflammatory response, immune response, and complement (Table 2). The genes represented by these categories included induced inflammatory genes, such as chemokines [chemokine (C-C motif) ligand 21], acute-phase proteins (SAA3, haptoglobin, and LBP), the LPS-signaling receptor (CD14 antigen), cytokine-associated genes (IL-10 receptor and IL-6 signal transducer), and reactive oxygen species (SOD2), in addition to the stress-responsive transcription factor signal transducer and activator of transcription-3 (STAT3) and antimicrobial milk proteins (such as lactoferrin; Table 2). Genes with functions related to cell proliferation and programmed cell death were also induced after a *Strep. uberis* infection with both apoptotic (dual-specificity phosphatase 6, growth arrest and DNA damage-inducible β , B-cell chronic lymphocytic leukemia/lymphoma 10) and antiapoptotic (SON DNA-binding protein) genes being induced. The genes represented in the cell proliferation category were primarily associated with the negative regulation of cell proliferation. The genes that were downregulated had functions related to lipid metabolism, vesicle-mediated transport, and cellular responses and cell death, including complement components (C1Q, C2, C6), cytokines (IL18), and transcription factors (signal transducer and activator of transcription 5A) and apoptotic serum glucocorticoid kinase (Table 3).

Eleven immune-associated genes that were differentially expressed by microarray analysis were also assessed by qRT-PCR (Figure 1a). Similar to the microarray analysis, C3, which was represented by 4 EST on the microarray, was significantly increased by 2.4-fold after *Strep. uberis* infection compared with noninfected samples. Clusterin and DAF are also associated with the complement cascade. The microarray analysis showed both clusterin (8 EST) and DAF (1 EST) had an increase in expression ($P < 0.05$) in infected tissue by 1.7- and 1.3-fold, respectively, compared with noninfected samples. However, qRT-PCR analysis showed that clusterin tended to be increased ($P < 0.1$) by 3.4-fold and DAF mRNA levels were unchanged in mastitic samples compared with noninfected samples. The chemokines, CXCL1 (1 EST) and IL-8 (1 EST) were downregulated ($P < 0.05$) in infected tissues by 1.8- and 1.5-fold, respectively, compared with nonin-

Table 2. Genes induced in *Streptococcus uberis*-infected tissue selected and identified to be of interest on the basis of their gene ontology terms

RefSeq identifier	Gene description	Gene symbol	Signed fold change
Programmed cell death			
NM_138927	SON DNA-binding protein	SON	7.26
NM_001423	Epithelial membrane protein 1	EMP1	2.67
NM_001946	Dual-specificity phosphatase 6	DUSP6	2.17
NM_015675	Growth arrest and DNA damage inducible β	GADD45B	2.04
NM_014456	Programmed cell death 4	PDCD4	1.87
NM_003921	B-cell chronic lymphocytic leukemia/lymphoma 10	BCL10	1.71
Reactive oxygen			
NM_000636	Superoxide dismutase 2	SOD2	5.90
NM_005410	Selenoprotein P	SEPP1	1.73
Inflammatory response			
NM_005621	S100 calcium-binding protein A12 (calgranulin C)	S100A12	7.26
NM_001979	Epoxide hydrolase 2	EPHX2	3.18
NM_000176	Glucocorticoid receptor	NR3C1	2.38
NM_138931	B-cell chronic lymphocytic leukemia/lymphoma 6 (zinc finger protein 51)	BCL6	2.35
NM_002306	Lectin, galactoside-binding	LGALS3	1.91
NM_000591	Cluster of differentiation molecule	CD14	1.75
NM_006908	Ras-related C3 botulinum toxin substrate 1	RAC1	1.67
NM_002989	Chemokine (C-C motif) ligand 21	CCL21	1.66
NM_002965	S100 calcium-binding protein A9 (calgranulin B)	S100A9	1.61
Acute-phase response			
NM_181016	Serum amyloid A3	SAA3	6.91
NM_005143	Haptoglobin	HP	2.37
NM_004139	LPS-binding protein	LBP	2.00
NM_139276	Signal transducer and activator of transcription 3	STAT3	1.69
Cell proliferation			
NM_006887	Zinc finger protein 36, C3H type-like 2	ZFP36L2	3.41
NM_005620	S100 calcium-binding protein A11 (calgizzarin)	S100A11	2.10
NM_003884	p300/cAMP response element binding (CREB)-binding protein (CBP) associated factor	PCAF	1.92
NM_032595	Protein phosphatase 1	PPP1R9B	1.89
NM_052987	Cyclin-dependent kinase 10	CDK10	1.73
NM_005981	Tetraspanin 31	SAS	1.71
NM_033222	RNA polymerase II transcription cofactor 4 (PC4) and splicing factor, arginine/serine rich interacting protein 1 (SFRS1)	PSIP2	1.61
Immune response			
NM_007074	Coronin, actin-binding protein, 1A	CORO1A	8.75
NM_021181	Signaling lymphocytic activation molecule (SLAM) family member 7	SLAMF7	6.45
NM_001814	Cathepsin C	CTSC	3.61
NM_002343	Lactotransferrin	LTF	3.39
NM_000631	Neutrophil cytosolic factor 4	NCF4	3.01
NM_000593	ATP-binding cassette, subfamily B	TAP1	2.83
NM_002123	Major histocompatibility complex, class II, DQ β 1	HLA-DQB1	2.73
NM_003618	Mitogen-activated protein kinase 3	MAP4K3	2.56
NM_172088	Tumor necrosis factor (ligand) superfamily, member 13	TNFSF13	2.50
NM_013416	Neutrophil cytosolic factor 4	NCF4	2.31
NM_002032	Ferritin	FTTH1	2.28
NM_007040	Heterogeneous nuclear ribonucleoprotein U-like 1	E1B-AP5	2.25
NM_001549	IFN-induced protein with tetratricopeptide repeats	IFIT4	2.19
NM_021109	Thymosin, β 4	TMSB4X	2.16
NM_004559	Y box-binding protein 1	NSEP1	2.15
NM_005180	B lymphoma Moloney murine leukemia virus (Mo-MLV) insertion region	BMI1	2.14
NM_002800	Proteasome subunit, β type, 9	PSMB9	1.99
NM_004838	Homer homolog 3 (<i>Drosophila</i>)	HOMER3	1.95
NM_004355	Cluster of differentiation 74 molecule, major histocompatibility complex, class II	CD74	1.92
NM_001558	IL-10 receptor	IL10RA	1.91
NM_002198	IFN regulatory factor 1	IRF1	1.91
NM_004048	β -2-microglobulin	B2M	1.90
NM_000570	Fragment crystallizable region of IgG, low affinity IIIb, receptor	FCGR3B	1.88
NM_139068	Mitogen-activated protein kinase 9	MAPK9	1.85
NM_002974	Serpin peptidase inhibitor	SERPINB4	1.85
NM_000565	IL-6 receptor	IL6R	1.83
NM_144646	IgJ polypeptide	IGJ	1.81

Continued

Table 2 (Continued). Genes induced in *Streptococcus uberis*-infected tissue selected and identified to be of interest on the basis of their gene ontology terms

RefSeq identifier	Gene description	Gene symbol	Signed fold change
NM_002177	Omega 1	IFNW1	1.80
NM_001560	IL-13 receptor	IL13RA1	1.75
NM_012420	IFN-induced protein with tetratricopeptide repeats	IFIT5	1.74
NM_019111	Major histocompatibility complex, class II, DR α	HLA-DRA	1.73
NM_002184	IL-6 signal transducer	IL6ST	1.73
NM_006016	Cluster of differentiation 164 molecule, sialomucin	CD164	1.72
NM_000637	Glutathione reductase	GSR	1.68
NM_152855	Ig λ -like polypeptide 1	IGLL1	1.65
NM_030781	Collectin subfamily member 12	COLEC12	1.64
NM_000377	Wiskott-Aldrich syndrome (eczema-thrombocytopenia)	WAS	1.62
NM_005615	Ribonuclease, RNase A family, k6	RNASE6	1.61
Complement			
NM_000064	Complement component 3	C3	2.36
NM_001710	Complement factor B	BF	1.95
NM_000574	Cluster of differentiation 55 molecule, decay-accelerating factor for complement	DAF	1.64

infected tissues by microarray analysis. In contrast, qRT-PCR analysis showed that CXCL1 mRNA levels were unchanged, although IL-8 was increased ($P < 0.05$) by 7.9-fold in infected tissue. Microarray analysis showed S100A12 was one of the most highly upregulated ($P < 0.05$) in *Strep. uberis*-infected tissue by 7.3-fold, and qRT-PCR analysis showed a dramatic increase, by over 20-fold compared with noninfected samples. The microarray analysis showed an increase in genes involved in the acute-phase response and oxidative stress (Table 2). The average fold change for EST for SAA3 and LBP was 7.6- and 2.0-fold, respectively. In comparison, qRT-PCR analysis showed an increase in SAA3 mRNA levels by 55-fold and for LBP mRNA levels by 13-fold. The oxidative stress genes metallothionein and SOD2 were upregulated by 9.4- and 2.8-fold by microarray analysis and 4.6- and 11.7-fold by qRT-PCR analysis. Lactoferrin, a milk protein with known antimicrobial activity, was upregulated by 3.4-fold (22 EST) on the microarray and 6.5-fold by qRT-PCR analysis.

Six different cytokines not represented on the microarray were also analyzed by qRT-PCR. Interleukin-1 β , IL-6, and IL-10 were all shown to be more abundant in *Strep. uberis*-infected tissue, by 15.9- ($P < 0.05$), 6.0- ($P < 0.05$), and 3.9-fold ($P < 0.1$), respectively (Figure 1b). The mRNA expression for TLR2, TNF- α , and IFN- γ also increased by 3.0- ($P < 0.05$), 3.4- ($P < 0.05$), and 2.4-fold ($P < 0.1$), respectively, in *Strep. uberis*-infected tissue (Figure 1b). Transforming growth factor- β and CXCL2 mRNA levels were the same in infected and noninfected samples.

The microarray analysis showed that the milk protein genes were downregulated after a *Strep. uberis* infection. The average downregulated fold change for the EST for α S1-CN was 1.4-fold (21 EST), α -LA was 1.6-fold (6 EST), β -LG was 1.3-fold (9 EST), β -CN

was 1.5-fold (12 EST), and κ -CN was 1.2-fold (8 EST). In comparison, qRT-PCR analysis showed that mRNA levels for α S1-CN, α -LA, β -LG, β -CN, and κ -CN were downregulated by 2.3-, 3.5-, 2.1-, 2.0-, and 1.6-fold, respectively, in *Strep. uberis*-infected compared with noninfected tissue (Figure 1c).

To further investigate the immune response induced by *Strep. uberis*, changes in mRNA levels of 9 selected immune-associated genes (C3, clusterin, IL-1 β , IL-6, IL-8, LAP, SAA3, S100A12, TLR2) were measured in bMEC after incubation with heat-killed bacterial cells (Figure 2). Interestingly, *Strep. uberis* 233 did not induce changes in expression of any of the genes in bMEC. In contrast, *E. coli* treatment induced a significant increase in expression of all of the genes in the epithelial cells, with the exception of clusterin (Figure 2). Challenging bMEC with an encapsulated strain of *Strep. uberis* (strain 262, a clinical isolate from a New Zealand farm), unlike *Strep. uberis* 233, caused a significant ($P < 0.05$) increase in expression of C3 (2.1-fold), SAA3 (4.8-fold), and IL-1 β (2.5-fold). The expression of these 3 genes, in addition to TLR2, was also increased ($P < 0.05$) 2.1-, 8.5-, 5.3-, and 1.7-fold, respectively, after incubation of bMEC with *Staph. aureus*. This increase was more than for *Strep. uberis* 262 but less than that observed for *E. coli* ($P < 0.05$). No change in expression was observed for clusterin for any of the bacterial stimuli.

DISCUSSION

This study has used microarray analysis to examine gene expression profiles associated with the response to *Strep. uberis* infections in the bovine mammary gland. In addition, immune responses to bacterial infection in the mammary gland (in vivo) and bMEC (in vitro) were compared by qRT-PCR. *Streptococcus uberis* infection

Table 3. Genes downregulated in infected tissue selected and identified to be of interest on the basis of their gene ontology terms

RefSeq identifier	Gene description	Gene symbol	Signed fold change
Lipid metabolism/biosynthesis and transport			
NM_000384	Apolipoprotein B	APOB	-3.20
NM_015533	Dihydroxyacetone kinase 2 homolog (<i>Saccharomyces cerevisiae</i>)	DKFZP586B1621	-2.97
NM_006227	Phospholipid transfer protein	PLTP	-2.55
NM_005928	Milk fat globule-epidermal growth factor 8 protein	MFGE8	-2.45
NM_147161	Acyl-coenzyme A thioesterase 11	THEA	-2.10
NM_002004	Farnesyl diphosphate synthase	FDPs	-2.10
NM_006579	Emopamil-binding protein	EBP	-2.07
NM_002661	Phospholipase C	PLCG2	-2.03
NM_017436	α 1,4-Galactosyltransferase	A4GALT	-2.01
NM_000697	Arachidonate 12-lipoxygenase	ALOX12	-1.99
NM_000483	Apolipoprotein C-II	APOC2	-1.96
NM_005084	Phospholipase A2, group VII	PLA2G7	-1.95
NM_005028	Phosphatidylinositol-4-phosphate 5-kinase, type II, α	PIP5K2A	-1.92
NM_000431	Mevalonate kinase	MVK	-1.92
NM_002335	Low-density lipoprotein receptor-related protein 5	LRP5	-1.78
NM_003708	Retinol dehydrogenase 16	RODH-4	-1.77
NM_032574	Dumpy (dpy)-30-like protein	LOC84661	-1.77
NM_002130	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	HMGCS1	-1.68
NM_001643	Apolipoprotein A-II	APOA2	-1.66
NM_002130	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	HMGCS1	-1.66
NM_145693	Lipin 1	LPIN1	-1.61
Cell death			
NM_005627	Serum/glucocorticoid-regulated kinase	SGK	-4.35
NM_001279	Cell death-inducing DNA fragment factor, 45kDa (DFFA)-like effector a	CIDEA	-2.77
NM_000875	IGF 1 receptor	IGF1R	-2.57
NM_003300	Tumor necrosis factor receptor-associated factor 3	TRAF3	-2.30
NM_002015	Forkhead box O1A	FOXO1A	-2.09
NM_020126	Sphingosine kinase 2	SPHK2	-1.88
NM_016222	Diethyl azodicarboxylate (Asp-Glu-Ala-Asp) box polypeptide 41	DDX41	-1.78
NM_000546	Tumor protein p53	TP53	-1.71
NM_001767	Cluster of differentiation 2 molecule	CD2	-1.69
NM_004295	Tumor necrosis factor receptor-associated factor 4	TRAF4	-1.69
NM_020529	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor	NFKBIA	-1.67
NM_002715	Protein phosphatase 2	PPP2CA	-1.66
NM_005671	Ultrabithorax domain	D8S2298E	-1.66
NM_001065	Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	-1.65
NM_018947	Cytochrome c	CYCS	-1.64
Protein transport			
NM_005057	Retinoblastoma-binding protein 5	RBBP5	-2.69
NM_007357	Component of oligomeric Golgi complex 2	COG2	-2.53
NM_006380	Amyloid β precursor protein-binding protein 2	APPBP2	-2.38
NM_005855	Receptor (calcitonin) activity-modifying protein 1	RAMP1	-2.20
NM_003302	Thyroid hormone receptor interactor 6	TRIP6	-2.08
NM_016128	Coatmer protein complex	COPG	-1.93
NM_004827	ATP-binding cassette, subfamily G	ABCG2	-1.92
NM_006178	N-Ethylmaleimide-sensitive factor	NSF	-1.84
NM_004663	Member Ras oncogene family	RAB11A	-1.84
NM_001442	Fatty acid-binding protein 4	FABP4	-1.83
NM_031431	Component of oligomeric Golgi	COG3	-1.75
NM_000498	Cytochrome P450, family 11, subfamily B, polypeptide 2	CYP11B2	-1.75
NM_018358	ATP-binding cassette, subfamily F	ABCF3	-1.74
NM_004102	Fatty acid-binding protein 3	FABP3	-1.72
NM_173471	Solute carrier family 25, member 26	SLC25A26	-1.72
NM_004371	Coatmer protein complex, subunit α	COPA	-1.67
NM_003938	Adaptor-related protein complex 3, Δ 1 subunit	AP3D1	-1.64
Vesicle-mediated transport			
NM_006370	Vesicle transport through interaction with t-soluble N-ethylamide-sensitive factor-attachment protein receptor (SNARE) 1B	VTI1B	-2.16
NM_006420	ADP-ribosylation factor guanine nucleotide exchange factor	ARFGEF2	-2.08
NM_003909	Copine III	CPNE3	-2.00
NM_004283	Member Ras oncogene family	RAB3D	-1.65
NM_007259	Vacuolar protein sorting 45A	VPS45A	-1.64

Continued

Table 3 (Continued). Genes downregulated in infected tissue selected and identified to be of interest on the basis of their gene ontology terms

RefSeq identifier	Gene description	Gene symbol	Signed fold change
Signaling			
NM_006988	A disintegrin and metalloprotease (ADAM)	ADAMTS1	-3.12
NM_006852	metallopeptidase with thrombospondin type 1		
NM_004180	Tousled-like kinase 2	TLK2	-2.69
	Tumor necrosis factor receptor-associated factor family member-associated nuclear factor κ B activator	TANK	-2.08
NM_000210	Integrin, α 6	ITGA6	-1.99
Cellular response			
NM_000676	Adenosine A2b receptor	ADORA2B	-2.17
NM_000631	Neutrophil cytosolic factor 4	NCF4	-2.17
NM_001562	IL-18	IL18	-1.79
NM_007146	Zinc finger protein 161	ZNF161	-1.79
NM_015991	Complement component 1, q subcomponent	C1Q	-1.74
NM_003152	Signal transducer and activator of transcription 5A	STAT5A	-1.68
NM_000065	Complement component 6	C6	-1.67
NM_000063	Complement component 2	C2	-1.65
NM_001716	Burkitt lymphoma receptor 1	BLR1	-1.62
NM_130386	Collectin subfamily member 12	COLEC12	-1.61

was associated with the differential expression of over 2,200 genes by >1.5-fold. Many of the genes with the greatest changes in expression had functions related to immune responses, demonstrating a clear upregulation of immune-associated genes after the onset of *Strep. uberis*-induced clinical mastitis. Utilization of bMEC cultures for analyzing immune gene regulation has demonstrated the complexity of the immune response of epithelial cells to different types and strains of bacterial infections.

Marked induction of genes encoding the acute-phase proteins SAA3, haptoglobin, and LBP were observed by microarray analysis or qRT-PCR analysis, or both, in *Strep. uberis*-infected tissue. Increased concentrations of SAA3 and haptoglobin and LBP have previously been reported in milk and serum, respectively, after a *Strep. uberis* infection (Pedersen et al., 2003; Bannerman et al., 2004a). In addition, LAP (a member of the major antimicrobial peptide family of defensins) is also increased in *Strep. uberis*-infected tissue (Swanson et al., 2004). Furthermore, the expression of LAP and SAA3 is increased after the infusion of LPS (the principal immunostimulatory molecule of the cell wall of gram-negative bacteria) in teat canals and by challenging primary bMEC in culture with LPS (Wellnitz and Kerr, 2004) and sonicated *E. coli* extracts (Stockum et al., 2008). Although LBP has primarily been associated with gram-negative bacterial infections, it has been demonstrated that LBP can also bind lipoteichoic acid, a component of the gram-positive bacterial cell wall (Schroder et al., 2003). Moreover, the LBP response to gram-positive bacteria was found to involve CD14,

with this pathway dependent on TLR2 (Schroder et al., 2003). In the present study, the CD14 molecule was also found by microarray analysis to be upregulated in *Strep. uberis*-infected tissue, and TLR2 was shown to increase significantly by qRT-PCR analysis. These observations suggest that the acute-phase proteins have a role in the immune response to *Strep. uberis* mastitis. Furthermore, the expression of a SAA3 homolog by bMEC (Molenaar et al., 2000) suggests at least some of these acute-phase proteins are part of a local inflammatory response.

The induction of apoptosis by bacterial pathogens is a well-established cellular process (Weinrauch and Zychlinsky, 1999). This microarray analysis demonstrated that genes associated with both apoptotic and antiapoptotic genes were upregulated after the onset of *Strep. uberis* mastitis. There is a loss of milk yield associated with mastitis, and this was further demonstrated in the present study by a downregulation in milk protein genes. This loss in milk yield could also be attributed to the loss of bMEC during *Strep. uberis* mastitis (Singh et al., 2006). This has also previously been demonstrated in both *Staph. aureus* and *E. coli* mastitis (Bayles et al., 1998; Long et al., 2001). The transcription factor, STAT3, was upregulated in the *Strep. uberis*-infected tissue on the microarray. In addition to being a mediator of the acute-phase response, STAT3 is also an essential mediator of apoptosis in the mouse mammary gland (Chapman et al., 1999; Humphreys et al., 2002). In agreement, STAT3 is activated during *Strep. uberis*-induced mastitis and is associated with an increase in bMEC apoptosis (Singh et al.,

Figure 1. Quantitative PCR analysis of selected genes showing the fold change in *Streptococcus uberis*-infected tissue. (+ P < 0.1; * P < 0.05; ** P < 0.01; *** P < 0.001). (a) Immune-associated genes represented on the microarray; (b) Immune-associated genes not present on the microarray; (c) Milk protein genes. Error bars denote SEM. C3 = complement component 3; CXCL1 = chemokine (C-X-C motif) ligand 1; DAF = decay-accelerating factor for complement; S100A12 = calgranulin C; SAA3 = serum amyloid A3; LBP = LPS-binding protein; MT = metallothionein; SOD = superoxide dismutase; CXCL2 = chemokine (C-X-C motif) ligand 2; TGF- β = transforming growth factor- β ; TLR2 = toll-like receptor 2; TNF- α = tumor necrosis factor- α .

2006). Although mastitis induced by *E. coli* resulted in increased bMEC apoptosis, an increase in genes associated with cell proliferation was also observed and may play a role in the tissue repair mechanism (Long et al., 2001; Capuco et al., 2003). In the present study, the up-regulation of antioxidants such as SOD2, selenoprotein P, and metallothionein also demonstrates a mechanism to limit tissue damage by phagocytosing polymorphonuclear neutrophil granulocytes, which actively release toxic oxygen radicals that not only kill the bacteria but also damage the host cells (Ward, 1983; Paape et al., 2003). Furthermore, the glycoprotein clusterin was increased in *Strep. uberis*-infected tissue. Studies in

vitro have demonstrated that clusterin is induced by reactive oxygen species (Viard et al., 1999). Clusterin has recently been shown to inhibit apoptosis (Zhang et al., 2005). This suggests that along with the proapoptotic and damage repair mechanism genes, there are also genes that may help regulate the process of apoptosis. Furthermore, the processes involving mastitis-induced apoptosis show similarity to those with normal mammary involution-induced apoptosis (Singh et al., 2008).

Cytokines mediate several aspects of the innate immune response including immune cell recruitment, activation, differentiation, and downregulation (Bannerman et al., 2004a). As was found in previous studies investigating the immune response to *Strep. uberis* in milk (Bannerman et al., 2004a), the cytokines in this study, IL-1 β , IL-8, IL-10, TNF- α , and IFN- γ , were all shown by qRT-PCR analysis to significantly increase in *Strep. uberis*-infected tissue. The chemokines CXCL1 and CXCL2 were upregulated in LPS-induced mastitis in a murine epithelial cell line (Zheng et al., 2006). However, significant increases in their expression in *Strep. uberis*-infected tissue were not observed in this study. Both IL-8 and CXCL1 were demonstrated to be down-regulated in *Strep. uberis*-infected tissue by microarray analysis. However, both of these genes were represented by only 1 EST on the microarray, further highlighting the important application of the microarray as a discovery tool rather than of quantitative analysis and the requirement of validation of differentially expressed genes identified by microarray analysis by the more sensitive qRT-PCR analysis (Rajeevan et al., 2001). The mRNA expression of IL-6 was significantly upregulated in *Strep. uberis*-infected tissue. There are no previous reports of IL-6 in association with *Strep. uberis* mastitis, but previous studies have shown an increase in IL-6 mRNA expression in milk somatic cells of dairy cows experimentally challenged with either *E. coli* or *Staph. aureus* (Lee et al., 2006).

The identification of genes that are upregulated in response to infection could have potential as candidate markers for the early detection of mastitis. However, due to the use of whole tissue in the microarray experiment, the identification of the immune response elicited by the bMEC was undetermined. Mastitis is associated with infiltration of the mammary tissue by neutrophils, macrophages, and lymphocytes. These, together with a range of different cytokines, chemokines, and additional cellular defense mechanisms, play a pivotal role in the elimination of invading bacteria. However, the recruitment of these cells into the mammary gland results in changes to the cellular composition of the mammary tissue. This has to be considered when attributing changes in gene expression in the mammary

Figure 2. Quantitative PCR analysis of immune-associated genes showing the fold change in expression after incubation of primary mammary epithelial cells with PBS alone (control) or PBS with strains of heat-killed *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis* 233, or *S. uberis* 262 (* $P < 0.05$). Error bars denote SEM. C3 = complement component 3; LAP = lingual antimicrobial peptide; MSAA = serum amyloid A3; S100A12 = calgranulin C; TLR2 = toll-like receptor 2.

gland after mastitis. Recent studies have demonstrated that bMEC also play an important role in the immune defense against invading pathogens (Pareek et al., 2005; Strandberg et al., 2005; Zheng et al., 2006). Therefore, the immune response of primary bMEC to different mastitis pathogens was investigated. Heat-killed bacteria were added to primary mammary epithelial cells at a concentration of 10^9 cfu/mL. This concentration was chosen by measuring the expression of LAP mRNA after incubations with serial dilutions of *E. coli* (data not shown). This is a greater concentration than the typical bacterial concentration observed in vivo, which ranges from 10,000 to 1,000,000 cfu/mL (Kornalijnslipper et al., 2003; Rambeaud et al., 2003; Middleton et al., 2004). A 12-h incubation of the epithelial cells with heat-killed *Strep. uberis* 233 resulted in no significant increase in the expression of any of the 9 immune-related genes investigated by qRT-PCR. In contrast, *E. coli* induced a significant increase in the transcript level for 8 of 9 immune-related genes. Furthermore, incubation with *Staph. aureus* and the encapsulated *Strep. uberis* 262 also induced an increase in expression of some of the immune-related genes (IL-1 β , C3, SAA3, and TLR2). Previous studies using bMEC observed that *Strep. uberis* at the same concentrations as *E. coli* and *Staph. aureus* did not increase the mRNA expression of measured cytokines and chemokines in primary bMEC (Wellnitz et al., 2006). Interestingly, not only do different bacteria elicit a different immune response as discussed earlier, but our results indicate that different strains of *Strep. uberis* also may elicit different immune responses by epithelial cells and one contributing factor

may be the hyaluronic acid capsule. Support for this has been found in studies of different strains of *Streptococcus suis*, in which capsule expression appeared to be important for the activation of several proinflammatory cytokines in a whole blood culture system (Segura et al., 2006). However, further studies would be required to investigate the effect of the *Strep. uberis* hyaluronic capsule on epithelial-derived immune components in primary mammary epithelial cell culture. Numerous studies have also highlighted differences in the expression of immune factors being dependent on the infecting pathogen (Bannerman et al., 2004a,b; Strandberg et al., 2005). Although *Strep. uberis* 233 was able to cause clinical mastitis after infusion into mammary quarters of dairy cows, this strain did not induce the expression of any of the selected proinflammatory cytokines and other immune-related components in primary bMEC. Yet, previous studies have demonstrated the expression and localization of immune-related genes to bMEC (Goldammer et al., 2004; Swanson et al., 2004). These results, together with other studies (Wellnitz and Kerr, 2004; Wellnitz et al., 2006), indicate that the expression of epithelium-derived immune components may not be initiated by *Strep. uberis* 233 but by the immune-modulatory components of other immune cells in the mammary gland.

CONCLUSIONS

The microarray analyses described in this study demonstrated the complex physiological and cellular processes that occur in the bovine mammary gland

after a *Strep. uberis* infection. Furthermore, changes in immune-related gene expression by mammary epithelial cells appear to be pathogen-specific. Together these results highlight the complexity of the immune response to an infecting pathogen and indicate that a coordinated response exists between the resident, recruited, and inducible immune factors.

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