NOTE *Immunology*

The cell wall component lipoteichoic acid of *Staphylococcus aureus* induces chemokine gene expression in bovine mammary epithelial cells

Yoshio KIKU[1)], Yuya NAGASAWA[1)], Fuyuko TANABE[1)], Kazue SUGAWARA[1)], Atsushi WATANABE[1)], Eiji HATA[1)], Tomomi OZAWA[2)], Kei-ichi NAKAJIMA[3)], Toshiro ARAI[4)] and Tomohito HAYASHI[1)]*

[1)]*Hokkaido Research Station, National Institute of Animal Health, NARO, Sapporo, Hokkaido 062–0045, Japan*
[2)]*National Institute of Animal Health, NARO, Tsukuba, Ibaraki 305–0856, Japan*
[3)]*Hokkaido Agricultural Research Center, NARO, Sapporo, Hokkaido 062–8555, Japan*
[4)]*School of Veterinary Medicine, Nippon Veterinary and Life Science University, Musashino, Tokyo 180–8602, Japan*

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ABSTRACT. *Staphylococcus aureus* (SA) is a major cause of bovine mastitis, but its pathogenic mechanism remains poorly understood. To evaluate the role of lipoteichoic acid (LTA) in the immune or inflammatory response of SA mastitis, we investigated the gene expression profile in bovine mammary epithelial cells stimulated with LTA alone or with formalin-killed SA (FKSA) using cap analysis of gene expression. Seven common differentially expressed genes related to immune or inflammatory mediators were up-regulated under both LTA and FKSA stimulations. Three of these genes encode chemokines (IL-8, CXCL6 and CCL2) functioning as chemoattractant molecules for neutrophils and macrophages. These results suggest that the initial inflammatory response of SA infection in mammary gland may be related with LTA induced chemokine genes.

KEY WORDS: bovine mammary epithelial cell, cap analysis of gene expression, chemokine, lipoteichoic acid, *Staphylococcus aureus*

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Bovine mastitis involves inflammation of the mammary gland and is commonly caused by bacterial infection [27]. Despite extensive management practices, it continues to be an economically important disease of dairy ruminants worldwide, owing to reduced milk yield, loss of milk that must be discarded after treatment, and the high cost of veterinary services. The gram-positive bacterium, *Staphylococcus aureus (S. aureus)*, is the primary cause of typical bovine mastitis ranging between the clinical and sub-clinical stages, through infection of the mammary tissue [26]. *S. aureus* infection often causes chronic inflammation in bovine mammary glands for the entire life of dairy cattle, sometimes without visible signs of disease. Upon bacterial infection of the mammary tissues, the host must be able to immediately initiate elimination processes [18]. At the early stages of infection, the predominant defense strategy that is rapidly induced is the innate immune response. In response to damage or the presence of invading pathogens, a variety of host cells, such as monocytes, macrophages and epithelial cells, produce and secrete potent immune and inflammatory mediators. Most of the pathogenic studies conducted to date have focused on bovine mastitis caused by *Escherichia coli* infection and the role of its major virulent factor, lipopolysaccharide (LPS); however, there is a lack of information on the pathogenic mechanism of *S. aureus* in bovine mastitis, despite its recognized importance in the dairy industry. Among the virulence factors of *S. aureus*, the cell wall component lipoteichoic acid (LTA) has been shown to play a pathogenic role in infectious diseases [3] through its involvement in biofilm formation, which promotes bacterial adherence to the host [4, 7]. Recent studies have investigated the transcriptional response to killed or inactive gram-positive pathogens and evaluated the contribution of gram-positive cell wall constituents, such as LTA, to the triggering of specific host defense responses [4, 16]. Although *S. aureus* has been considered a major pathogenic bacterium of bovine mastitis, it has thus far been difficult to identify the exact role of the individual bacterial cell components, including LTA, in relation to the onset of mastitis.

Studies evaluating the responses to infections at various epithelial sites strongly suggest that epithelial cells are capable of responding to bacterial intrusion, suggesting that they play a major role in the initiation of inflammation [1, 16]. Indeed, in responding to microbial infection, intestinal [12] and respiratory [1, 16] epithelial cells are well known to initiate the recruitment of neutrophils through their inflammatory and immune responses. Kang *et al.* suggested that *S. aureus* stimulates the human intestinal epithelial cells to induce the chemokine interleukin (IL)-8 production through its LTA, potentially contributing to the development of intestinal inflammation [12]. Based on this background, we considered whether bovine mammary epithelial cells (BMECs) could act as sentinels for signal invading mastitis-causing pathogens. Therefore, we investigated the role of LTA on the early immune responses of BMECs against *S. aureus* infection.

To reveal whether LTA of *S. aureus* could induce immune or inflammatory response mediators in mastitis, we deter-

*CORRESPONDENCE TO: HAYASHI, T., Hokkaido Research Station, National Institute of Animal Health, NARO, 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062–0045, Japan.
e-mail: hayatomo@affrc.go.jp

mined the gene expression profile of BMECs stimulated with purified LTA alone or formalin-killed *S. aureus* (FKSA) constituents containing all bacterial components, including LTA, using cap analysis of gene expression (CAGE) [13]. This technique has great advantages compared to classical microarray-based expression detection techniques [11, 13]. Specifically, CAGE can be identified the DNA regulatory elements that are specific for biological phenomenon by looking at the sequences that are in the promoters of the RNA isoforms being expressed in the analyzed samples. This technique also enables identification of transcriptional start sites (TSSs) of differentially expressed genes (DEGs) that are related to the stimulus. The expression level of each DEG was quantified, including annotated genes, in the target cells under the induction conditions to mimic bovine mastitis. The use of the CAGE technology is considered to be useful for the development of new diagnostics and therapy of bovine mastitis.

Purified LTA derived from *S. aureus* was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.), which was adjusted to 1 μg/m*l* in fetal bovine serum FBS-free fresh Dulbecco's modified Eagle medium (DMEM) for LTA stimulation. The *S. aureus* strain BM1006, which was originally isolated from a cow at a Japanese dairy farm, was used for preparation of FKSA. Multilocus sequence typing analysis revealed that strain BM1006 was sequence type 352 (ST352), which is one of the most common isolates from *S. aureus* strains causing bovine mastitis [9, 22]. The washed *S. aureus* BM1006 was suspended in phosphate-buffered saline containing 0.5% formaldehyde, incubated overnight at room temperature for inactivation and then washed 3 times by PBS. Finally, the killed *S. aureus* was suspended in DMEM and adjusted to 2.5×10^8 cells/m*l* for FKSA stimulation.

The procedure for isolation of BMEC clones was performed as described by Nakajima *et al.* [17]. The cloned BMECs were maintained in DMEM supplemented with 10% (vol/vol) FBS, 5 μg/m*l* of insulin, 50 U/m*l* of penicillin and 50 μg/m*l* of streptomycin in 25-cm^2 culture flasks. When the BMECs reached 80% confluence, fresh FBS-free DMEM was added to the flasks with or without LTA or FKSA. After incubation of the BMECs with these stimulators for 6 hr, the cells were harvested by directly adding 520 μl of lysis buffer to each flask according to the manufacturer's protocol of Quick Gene RNA cultured cell Kit S (FUJIFILM Corp., Tokyo, Japan). After completion of BMEC lysis, total RNA was extracted from the BMECs using the Quick Gene RNA cultured cell Kit S and nucleic acid isolation system QG-810 (FUJIFILM Corp.).

The CAGE library was prepared following the protocol described by Kodzius *et al.* [13], which was modified by using adaptors suitable for direct sequencing on an Illumina GAII platform; the adaptors were prepared and obtained from DNAFORM (Yokohama, Japan). In brief, LTA- and FKSA-stimulated BMEC complementary DNA (cDNA) was synthesized from total RNA using a mixture of random and oligo-dT primers using PrimerScript RT Master Mix (TAKARA BIO INC., Tokyo, Japan) according to the manufacturer's protocol. The 5′ end of cDNA was selected using the cap-trapper method, and cDNA was ligated to a linker containing a recognition site for *Eco*P15I. After the second strand was synthesized, *Eco*P15I cleaved the cDNAs at a site 27 nucleotides away from the 5′ end to produce the CAGE tags. Next, a linker was attached to the 3′ end of the tag sequence for amplification. Sequencing of the CAGE library was performed on a Genome Analyzer II platform (Illumina, San Diego, CA, U.S.A.). Mapping of CAGE-tag sequences to the bovine genome (NCBI Btau 4.0) was performed at Genomatix (Genomatix Software, München, Germany). We used cluster analysis for genome-wide identification of DEGs, by determining the local enrichments of CAGE tags representing TSSs obtained from Genomatix. All TSS clusters were correlated with transcripts annotated in the ElDorado database (Genomatix, NCBI Btau 4.0 Version 07–2009).

First, we analyzed DEGs in the profile of BMECs stimulated with LTA alone according to the CAGE results. A total of 59,441 TSSs of DEGs were induced by the LTA stimulation, including 41 up-regulated and 141 down-regulated genes. Furthermore, 29 of the 41 up-regulated DEGs and 79 of the 141 down-regulated DEGs could be annotated to known genes. After filtering DEGs showing log FC >0.61 and an adjusted *P*-value <0.05, we found 5 immune response-related DEGs (*IL-8, CXCL6, RSAD2, CCL2* and *IL-1α*) and 5 inflammatory response-related DEGs (*IL-8, CXCL6, CCL2, IL-1α* and *NFKBIZ*) among the 41 up-regulated genes. Table 1 shows the top 20 up-regulated DEGs with the highest expression levels. The remaining up-regulated DEGs were not annotated to genes related to either immune or inflammatory responses in the BMEC genome. The CAGE results were confirmed using real-time RT-PCR of *IL-8* and *CXCL6* genes. Their expression levels after the stimulation of LTA were 17.1-fold and 22.2-fold of a control, respectively (data not shown). On the other hand, of the 141 down-regulated DEGs, after filtering with the criteria of DEGs showing a log FC >−1.42 and adjusted *P*-value<0.05, no immune response- or inflammatory response-related DEGs were identified (data not shown).

Next, we analyzed the DEG profile in the BMECs stimulated with FKSA according to the CAGE results. A total of 57,417 TSSs of DEGs were induced by the FKSA stimulation, including 54 up-regulated genes and 15 down-regulated genes. Furthermore, 29 of the 54 up-regulated genes and 4 of the 15 down-regulated genes could be annotated with known DEGs. Table 2 shows the top 20 up-regulated genes with the highest expression levels. After filtering the up-regulated DEGs with the criteria of log FC >0.64 and adjusted *P*-value<0.05, we found 6 immune response-related DEGs (*IL-8, CXCL6, ERAP2, CCL2, IL-1α* and *BCL3*) and 6 inflammatory response-related DEGs (*IL-8, SPP1, CXCL6, CCL2, IL-1α* and *NFKBIZ*). The remaining genes were not annotated to immune or inflammatory response-related genes in the BMEC genome. The CAGE results were confirmed using real-time RT-PCR of *IL-8* and *CXCL6* genes. Their expression levels after the stimulation of FKSA were 10.4-fold and 8.3-fold of a control, respectively (data not shown). Similar to the results for LTA stimulation alone,

Table 1. Top 20 up-regulated genes[a] in bovine mammary epithelial cells stimulated with lipoteichoic acid

Gene symbol	Gene name	Gene accession	Immune response	Inflammatory response	Log FC	*P*-value[b]	Common with FKSA[c] stimulation
IL8	interleukin 8, chemokine (C-X-C motif) ligand 8 (CXCL8)	NM_173925.2	Yes	Yes	6.1	3.20E-25	Yes
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_174300.2	Yes	Yes	4.14	6.10E-78	Yes
MCM7	minichromosome maintenance complex component 7	NM_001025345.2			3.66	0.03	
MX2	myxovirus (influenza virus) resistance 2 (mouse)	NM_173941.2			2.73	0.0089	Yes
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_001045868.1			1.53	0.0065	
RSAD2	radical S-adenosyl methionine domain containing 2	NM_001045941.1	Yes		1.43	0.0089	
CCL2	chemokine (C-C motif) ligand 2	NM_174006.2	Yes	Yes	1.37	3.90E-13	Yes
IL1A	interleukin 1, alpha	NM_174092.1	Yes	Yes	1.2	0.0019	Yes
MOV10	Mov10 RISC complex RNA helicase	NM_001075839.1			1.19	0.017	
PRKCSH	protein kinase C substrate 80K-H	NM_176662.1			0.96	0.028	
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	NM_174726.1		Yes	0.92	8.80E-07	Yes
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_174445.2			0.88	0.0021	Yes
RPS2	ribosomal protein S2	NM_001033613.1			0.86	0.033	
ZNF706	zinc finger protein 706	NM_001199073.1			0.8	0.022	
LOC504599	histone H3.2	NM_001166569.1			0.77	0.022	
KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	NM_001079779.1			0.74	0.023	
ADM	adrenomedullin	NM_173888.3			0.72	0.0065	
LOC505183	histone H2B type 1-like	XM_581429.4			0.66	0.024	
HIST1H2AC	histone cluster 1, H2ac	XM_603142.2			0.61	0.033	
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	NM_174267.2			0.61	0.0008	

a) Up-regulated genes showing a value greater than 1 log FC, defined as the logarithm of the gene expression level in a sample relative to that of the non-stimulated control. b) Significance of the difference in gene expression levels between the sample and control. c) Formalin-killed *Staphylococcus aureus*.

of the 15 down-regulated DEGs, after filtering for a log FC >−1.42 and adjusted *P*-value<0.05, no immune or inflammatory response-related DEGs were found (data not shown). Although the immune and inflammatory response mediators involved in the pathogenesis of *S. aureus in vivo* have not been elucidated, the results of this CAGE study identified 7 commonly up-regulated DEGs, *IL-8, CXCL6, MX2, CCL2, IL-1α, NFKBIZ* and *PTGS2*, in both the FKSA and LTA stimulations. Moreover, three of these encode chemokines (IL-8, CXCL6 and CCL2), and one encodes a cytokine (IL-1α).

Interleukin (IL)-8, also known as chemokine (C-X-C motif) ligand 8 (CXCL8), is a small cytokine belonging to the CXC chemokine family. IL-8 is a chemokine produced by macrophages and other cell types, such as epithelial cells, airway smooth muscle cells and endothelial cells [10, 15]. IL-8, also known as neutrophil chemotactic factor, has primary functions of inducing chemotaxis to target cells, not only primarily neutrophils but also other granulocytes, to promote their migration toward the site of infection in bovine mastitis [20, 24]. IL-8 also induces phagocytosis once arriving at the infection site. In addition, an increased level of IL-8 is often found in acute diseases, such as mastitis, caused by *E. coli*, as well as in the chronic inflammatory phase of sub-clinical dry-period mastitis [25]. CXCL6 is a member of the C-X-C motif chemokine family and is also reported to function as a chemoattractant for neutrophilic granulocytes. Previous studies have shown that CXCL6 levels are increased during mucosal inflammation (e.g., in inflammatory bowel disease), similar to the function of the structurally related chemokine IL-8 [14, 23]. In addition, there are common receptors on the cell surface membrane capable of binding to both IL-8 and CXCL6; for example, the G protein-coupled receptors, CXCR1 and CXCR2, show affinity to both IL-8 and CXCL6. These chemokines are secreted through a signaling cascade

Table 2. Top 20 up-regulated genes[a)] in bovine mammary epithelial cells following stimulation with formalin-killed *Staphylococcus aureus*

Gene symbol	Gene name	Gene accession	Immune response	Inflammatory response	Log FC	*P*-value[b)]	Common with LTA[c)] stimulation
IL8	interleukin 8,chemokine (C-X-C motif) ligand 8 (CXCL8)	NM_173925.2	Yes	Yes	4.92	1.90E-10	Yes
SPP1	secreted phosphoprotein 1	NM_174187.2		Yes	3.51	5.90E-39	
ANKRD37	ankyrin repeat domain 37	NM_001075392.1			3.07	0.0089	
CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_174300.2	Yes	Yes	2.74	1.60E-24	Yes
C29H11orf86	chromosome 29 open reading frame, human C11orf86	NM_001077090.1			2.7	0.035	
GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1	NM_175777.3			2.66	0.0035	
MX2	myxovirus (influenza virus) resistance 2 (mouse)	NM_173941.2			2.57	0.013	Yes
ATP6V1C1	ATPase, H+ transporting, lysosomal 42 kDa, V1 subunit C1	NM_176676.1			2.07	0.045	
ERAP2	endoplasmic reticulum aminopeptidase 2	NM_001075628.2	Yes		2.07	0.015	
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	NM_001192294.1			1.65	0.0043	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclo-oxygenase)	NM_174445.2			1.49	2.20E-12	Yes
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	NM_174267.2			1.11	4.90E-11	
CCL2	chemokine (C-C motif) ligand 2	NM_174006.2	Yes	Yes	1.07	0.013	Yes
IL1A	interleukin 1, alpha	NM_174092.1	Yes	Yes	1.02	0.032	Yes
C27H8orf4	chromosome 27 open reading frame, human C8orf4	NM_001035490.2			1.01	5.00E-09	
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	NM_174726.1		Yes	0.83	0.00019	Yes
DUSP6	dual specificity phosphatase 6	NM_001046195.1			0.78	8.40E-05	
MASTL	microtubule associated serine/threonine kinase-like	NM_001113765.1			0.71	0.011	
SIK1	salt-inducible kinase 1	XM_003581774.1			0.67	0.0024	
BCL3	B-cell CLL/lymphoma 3	NM_001205993.1	Yes		0.64	0.023	

a) Up-regulated genes showing a value greater than 1 log FC, defined as the logarithm of the gene expression level in a sample relative to that of the non-stimulated control.b) Significance of the difference in gene expression levels between the sample and control. c) Lipoteichoic acid.

of ligand/receptor reactions and serve as important mediators of the immune reaction in the innate immune system response. A previous report also shows that intramammary infusion of LTA induced the secretion of IL-8 in milk [19]. Therefore, our findings suggest that the up-regulated IL-8 and CXCL6 in response to stimulation by whole *S. aureus*, including LTA, might function as immune and inflammatory mediators in *S. aureus*-induced mastitis.

CCL2 is a member of the C-C motif chemokine family and has been reported to recruit monocytes, memory T cells and dendritic cells to the sites of inflammation produced by either tissue injury or infection. CCL2 is secreted upon stimulation by monocytes and other innate cells [2]. In response to the CCR2-CCL2 interaction, monocytes are trafficked to the sites of microbial infection [21]. Monocytes differentiate into macrophages or dendritic cells to curtail the infection by directly phagocytizing and killing the pathogens. Thus, monocytes, along with neutrophils, form an integral part of the innate immune system and play a key role in the early containment of infections, such as mastitis.

IL-1α is mainly produced by activated macrophages, as well as neutrophils, epithelial cells and endothelial cells. It shows metabolic, physiological and hematopoietic activities and plays a central role in regulation of the immune response. IL-1α binds to the IL-1 receptor and is involved in the pathway that activates tumor necrosis factor-alpha (TNF-α). A previous study showed that BMECs stimulated with heat-inactivated preparations of *E. coli* displayed coordinated gene regulation governed by the activation of IL-1α and TNF-α signaling; however, this appears to be an *E. coli*-specific immune response feature, because stimulation of BMECs with *S. aureus* did not significantly alter IL-1α [8]. Similarly, other studies indicated that stimulation of BMECs with LTA did not significantly alter the expression of Toll-

like receptor pathway genes, including *IL-8* and *CXCL6*, or interferon-inducible genes, including *CCL2* [6, 23]. However, the results of the present CAGE study demonstrated that 4 of the up-regulated DEGs (*IL-8, CXCL6, CCL2* and *IL-1α*) were common to both the FKSA and LTA stimulations of BMECs. Thus, using CAGE, we were able to clarify the indeterminate expression of chemokine genes that have not been previously identified under induction conditions mimicking bovine mastitis. In addition, 5 DEGs expressed under both LTA and FKSA stimulations were associated with the inflammatory response, including *IL-8, CXCL6, CCL2, IL-1α* and *NFKBIZ*. In particular, IL-8 and CXCL6 are predicted to play an important role in this response given their roles as chemoattractant for neutrophilic granulocytes, as described above. Therefore, our findings imply that the up-regulated DEGs observed in response to both LTA and FKSA were mainly induced by LTA stimulation. These results suggest that the initial inflammatory response of *S. aureus* infection in mammary gland may be related with LTA induced chemokine genes. Further consideration will be needed to yield any findings about the differences between the bacterial species causing mastitis.

The simultaneous detection of temporal expression patterns of pathogens and host cells, especially during *S. aureus* mastitis, will help to define the pathogenesis of mastitis and provide further insight into the molecular cross-talk between pathogens and host cells. The CAGE results described herein have revealed novel mechanisms of chemokine induction by *S. aureus* LTA.

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