

Transcriptomic response of goat mammary epithelial cells to *Mycoplasma agalactiae* challenge – a preliminary study*

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Mycoplasma agalactiae (*Ma*) is one of the main aetiological agents of intramammary infections in small ruminants, causing contagious agalactia. To better understand the underlying disease patterns a primary goat mammary epithelial cell (pgMEC) culture was established from the mammary tissue and challenged with *Ma*. High-throughput mRNA sequencing was performed to reveal differentially expressed genes (DEG) at different time-points (3 h, 12 h, and 24 h) post infection (PI). The pathway enrichment analysis of the DEG showed that infection significantly affected pathways associated with immune response, steroid metabolism, fatty acid metabolism, apoptosis signalling, transcription regulation, and cell cycle regulation. Based on the results we suggest that mammary epithelial cells *in vivo* contribute to the immune system by the induced expression of cytokines and other chemotactic agents, activation of the complement system and apoptosis pathways, and expression of genes coding for antimicrobial molecules and peptides. In our study we attempted to interpret the detected transcriptomic changes in a biological context and infer mammary infection

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resistance candidate genes, interesting for further validation. Additionally, the results represent comprehensive goat mammary transcriptome information and demonstrate the applicability of the comparative genomics approach for annotation of goat data, using transcriptome information of a closely related species (*Bos taurus*) as a reference.

KEY WORDS: cell culture / high-throughput RNA sequencing / mammary gland / mastitis / *Mycoplasma agalactiae*

Mammary epithelial cells represent the first line of defence against invading pathogens and play a key role in innate immune response during intramammary infections [Stelwagen *et al.* 2009]. In small ruminants, *Mycoplasma agalactiae* (*Ma*) is one of the main aetiological agents of intramammary infections, causing contagious agalactia (CA) [Bergonier *et al.* 1997]. Mycoplasmas cause DNA damage in the host cells by overexpressing endonucleases, leading to inhibition of cell proliferation and apoptosis [Sokolova *et al.* 1998]. Apart from ethical issues and variation introduced by the animal's individual traits [Burvenich *et al.* 2003], experimental treatments *in vivo* cause systemic effects, which hinders control of the environment or elucidation of the contribution of a particular cell type [Rose and McConochie 2006]. Cell-based models surmount this problem and enable physiological and immunological studies of particular cell type(s). In this preliminary study, next-generation sequencing was used to assess transcriptomic response of *Ma* challenged primary goat mammary epithelial cells (pgMEC) after 3, 12, and 24 h.

Material and methods

The mammary tissue was obtained from the slaughtered lactating Saanen goat (*Capra hircus*). The animal was examined by a veterinarian and showed no signs of clinical mastitis or other diseases. The swab sample was collected from the tracheal mucus of the slaughtered animal for tests on modified Frey's agar and broth to rule out the presence of subclinical infections caused by *Mycoplasma* species. The primary cell culture used in the study was prepared from the removed mammary tissue of the slaughtered animal and plated on Geltrex (Gibco) covered plastic flasks as described previously [Ogorevc *et al.* 2009b]. Luminal, myoepithelial and fibroblast cells were characterized using antibodies against cytokeratin 18 (sc-51582, Santa Cruz Biotechnology), cytokeratin 14 (PRB-155P, Covance) and vimentin (sc-73262, Santa Cruz Biotechnology), respectively. The cell line was enriched with epithelial cells, using differential trypsinization. Pathogenic *Ma* (PG2 strain) was grown [Kleven 2003] until the exponential phase of growth and used to infect (MOI of approx. 100) the first passage epithelial fraction-enriched pgMEC. To exclude possible contamination of the derived primary cell culture with *Mycoplasma spp.* and to confirm the presence of *Ma* in the infected samples PCR based detection of mycoplasma-specific DNA sequences was performed, using 16S ribosomal RNA universal primers (U4-F: 5'-

GTAGTCCACGCCGTAACG-3', U8-R: 5'-CACAGGAAACAGCTATGACC-3') [Johansson *et al.* 1998]. Total RNA was extracted from the triplicates of samples of non-infected cells and cells infected for 3, 12, and 24 h. A mRNA-Seq library was prepared (Illumina, protocol version 1004898 Rev. D) and sequenced in a total of five lanes in three single read flow cells (v2, 1.4 mm) on an Illumina Genome Analyzer (version IIx, SCS2.5/RTA). The Burrows-Wheeler Aligner (ver. 0.5.7, <http://bio-bwa.sourceforge.net/>) [Li and Durbin 2009] was used to map the 50 bp sequences against the *Bos taurus* reference sequences (NCBI-RefSeq) (ftp://ftp.ncbi.nih.gov/refseq/B_taurus/). Differential expression analysis was performed in the R statistical environment (version 2.11.0, <http://www.R-project.org>), using the Bioconductor package edgeR (version 1.6.5) [Robinson *et al.* 2010]. The quantile-adjusted conditional maximum likelihood (qCML) method, followed by an estimation of the moderated tagwise dispersions (using prior.n value of 10) was used. Genes with a false discovery rate (FDR) value of less than 0.05 [Benjamini and Hochberg 1995] were considered significantly affected. The de-multiplexed fastq files and the processed data are available at GEO (<http://www.ncbi.nlm.nih.gov/geo/>) [Barrett *et al.* 2005 and Edgar *et al.* 2002], using the accession number GSE30379. The pathway enrichment analysis was conducted in DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>).

Results and discussion

Digestion of mammary tissue resulted in a heterogeneous population of cells consisting of two predominant cell types – luminal and myoepithelial. A small proportion of basal/fibroblast-like cells was also observed. When grown on plastic surface, cobblestone morphology typical of epithelial cells (Fig. 1-A, a), and larger, irregularly shaped fibroblast-like cells were observed (Fig. 1-A, b). Myoepithelial cells were positive for cytokeratin 14, whereas luminal for cytokeratin 18 (Fig. 1-A, c-d).

The *Ma* infection caused moderate fold changes (FC) in the expression of the differentially expressed genes (DEG) and no visible phenotypic changes at the cellular level, which is consistent with chronic or subclinical course of contagious agalactia *in vivo* [Castro-Alonso *et al.* 2009]. The most expressed genes were various cytokeratins and actins. For example, in all of the sequenced samples cytokeratin 5 was found to be the most abundant with 146.404 (SD=10.454) detected reads on average (N=3), while for actin beta 11.681 (SD=1.209) reads were detected. Immune-related genes were not among the most expressed in terms of absolute expression at any of the time-points post infection (PI). Their expression ranged from approximately a hundred to several thousand detected reads per sample. However, they were among the most differentially expressed (mostly up-regulated) at 12 and 24 h PI (Fig. 2).

The largest change in the transcriptome profile was observed at 24 h PI with 1562 DEG, followed by 856 at 3 h PI, and only 113 at 12 h PI. This might be explained by two-phased advancement of the immune response, in which early response genes were triggered immediately after the infection and were degraded, while late response

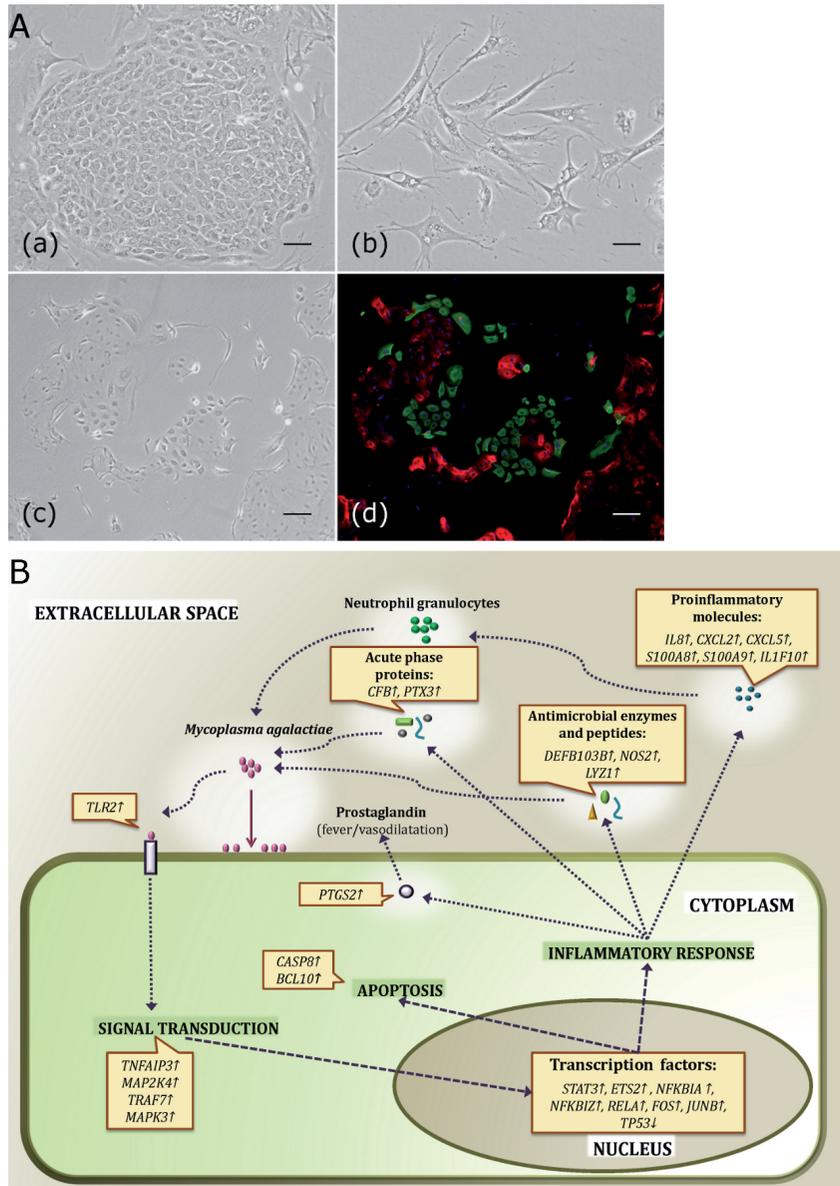


Fig. 1. A) Primary goat mammary cell culture. (a) An island of epithelial cells surrounded with basal/fibroblast-like cells, grown as a monolayer on Geltrex-covered surface. Scale bar = 100 μ m. (b) Fibroblast-like cells. Scale bar = 100 μ m. (c and d) Primary cell line under bright field (c) and fluorescent illumination (d), showing a mixed culture of cytokeratin 18 positive luminal epithelial cells (red) and cytokeratin 14 positive myoepithelial cells (green). The nuclei were counterstained with 4',6-diamidino-2-phenylindole - DAPI (blue). Scale bar = 100 μ m.

B) Recapitulation of possible pgMEC immune response mechanisms, estimated according to the differential gene expression of *Mycoplasma agalactiae* challenged cells. *BCL10* - B-cell CLL/lymphoma 10; *CASP8* - caspase 8, apoptosis-related cysteine peptidase; *CFB* - complement factor B; *CXCL2* - chemokine (C-X-C motif) ligand 2; *CXCL5* - chemokine (C-X-C motif) ligand 5; *DEFB103B* - defensin, beta 103B; *ETS2* - v-ets erythroblastosis virus E26 oncogene homolog 2 (avian); *FOS* - FBJ murine osteosarcoma viral oncogene homolog; *IL1F10* - interleukin-1 family member 10; *IL8* - interleukin 8; *JUNB* - jun B proto-oncogene; *LYZI* - lysozyme 1; *MAP2K4* - mitogen-activated protein kinase kinase 4; *MAPK3* - mitogen-activated protein kinase 3; *NFKBIA* - nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; *NFKBIZ* - nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta; *NOS2* - nitric oxide synthase 2, inducible; *PTGS2* - prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); *PTX3* - pentraxin 3; *RELA* - v-rel avian reticuloendotheliosis viral oncogene homolog A; *S100A8* - S100 calcium binding protein A8; *S100A9* - S100 calcium binding protein A9; *STAT3* - signal transducer and activator of transcription 3; *TLR2* - toll-like receptor 2; *TNFAIP3* - tumor necrosis factor, alpha-induced protein 3; *TP53* - tumor protein p53; *TRAF7* - TNF receptor-associated factor 6.

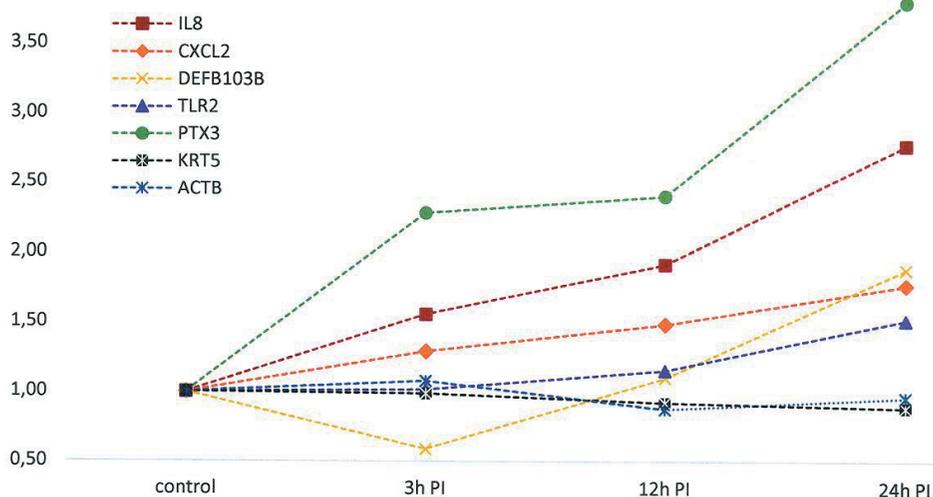


Fig. 2. Gene expression at different time-points post infection, presented as fold changes in relation to the control (FC = 1), for the immune-related genes (interleukin 8 - *IL8*; chemokine (C-X-C motif) ligand 2 - *CXCL2*; defensin, beta 103B - *DEFB103B*; toll-like receptor 2 - *TLR2*; and petraxin 3 - *PTX3*) that were significantly differentially expressed and for two of the most expressed genes in terms of absolute expression (cytokeratin 5 - *KRT5* and actin beta - *ACTB*) that were not significantly affected by the infection.

genes required more than 12 h to be regulated in response to the infection.

The pathway enrichment analysis of the DEG showed that the infection affected the immune response, steroid and fatty acid metabolism, apoptosis signalling, transcription regulation and cell cycle regulation. The pro-inflammatory signalling directed towards the induced expression of cytokines (*e.g.* *IL8*, CXC-chemokines) and other chemotactic agents (*e.g.* S100 calcium binding genes), genes coding for antimicrobial molecules and peptides (*e.g.* beta defensin – *DEFB103B*-like, lysozyme – *LYZI*, and nitric oxide synthases 2 – *NOS2*), and activation of complement system

pathways (e.g. complement factor beta – *CFB* and mannose-associated serine protease 1 – *MASPI*) was activated. Acute phase protein pentraxin 3 (*PTX3*) was also up-regulated.

The most significantly up-regulated cytokine was interleukin-8 (IL8), which was also up-regulated during intramammary infections with other pathogens [Bannerman *et al.* 2004; Gunther *et al.* 2011; Gunther *et al.* 2009; Mitterhuemer *et al.* 2010]. Based on the bioinformatic analyses it seems that IL8 acts as a node in a complex network, connecting inflammatory signalling and effector molecules. Additionally, when we compared the list of genes from Table 1 to the literature-collected list of promising candidate genes for mastitis resistance [Ogorevc *et al.* 2009a] we found *IL8* and *TLR2* on both lists, which further confirms their fundamental role in innate immunity of the mammary gland.

Interestingly, the most significantly up-regulated gene at 12 and 24 h PI (up-regulated 1.81 and 2.81-fold, respectively) was aquaporin 3 (*AQP3*). *AQP3* coordinated with *NOS2* were previously described as inflammatory mediators in the skin epithelia of new-born children [Marchini *et al.* 2003]. It is possible that the same (*AQP3-NOS2*) mechanism also plays a role in the innate immunity of the mammary gland, because both genes were among the most significantly up-regulated.

Structural genomic studies showed that goats are relatively closely related to bovine species [Schibler *et al.* 1998]. Additionally, bovine microarrays may be successfully used for cross-species hybridization in goats [Pisoni *et al.* 2010]. The use of a well-annotated bovine genome as an annotation reference for goat sequences seems justified, at least until a properly annotated caprine genome is available. Goat sequences that exhibited high similarity with bovine reference sequences were considered goat orthologs of the bovine genes. However, it is possible that due to insufficient sequence similarities between bovine and caprine homologs some of the reads were discarded in the process of mapping, although relative differences in expression between the samples were not affected by that fact. An additional problem in the case of farm animals is connected with bioinformatic analyses of candidate genes. Pathway analysis tools are mostly based on data obtained from human or mouse. Due to physiological and anatomical differences between species, a direct cross-species comparison of biological pathways and processes is not always justified. Thus, careful consideration and interpretation of the results is necessary when predicting enriched metabolic pathways in farm animals. Based on the results we can speculate that the immune contribution of the pgMEC *in vivo* is important for recruitment of neutrophils, activation of the complement system, expression of several bactericidal effector molecules, and apoptosis signalling (Fig. 1-B). Further studies and validation of candidate genes on a larger number of animals are necessary to compile data that will be representative of the immune contribution of luminal cells during intramammary infections. However, due to the lack of such studies using mycoplasmas we hope that this preliminary study could serve as a starting point to other researchers and that we illuminated some of the *Ma*-specific as well as general mammary immune response

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mechanisms in ruminants. Additionally, using bovine sequences as a reference we demonstrated the applicability of the comparative genomics approach to annotate data of species with poorly/un-annotated genome, using a well-annotated genome of a closely related species. Finally, the generated data also represents a wealth of publicly available goat transcriptome information.

Table 1. Representative *Mycoplasma agalactiae* immune response-related candidate genes

Gene Symbol*	Description	FC†	Assigned immune associated pathway(s)	FDR
			3 h PI	
<i>ATF3</i>	activating transcription factor 3	↓1.54	apoptosis signalling	0.01
<i>BCL2L1</i>	BCL2-like 1, nuclear gene encoding mitochondrial protein	↑1.65	p53 signalling, apoptosis signalling	0.00
<i>E2F1</i>	E2F transcription factor 1	↓1.73	p53 signalling	0.00
<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog	↑1.50	apoptosis signalling	0.00
<i>GADD45B</i>	growth arrest and DNA-damage-inducible, beta	↑1.51	p53 signalling	0.00
<i>MAP2K3</i>	mitogen-activated protein kinase 3	↑1.51	toll receptor signalling, apoptosis signalling	0.00
<i>PTGS2</i>	prostaglandin-endoperoxide synthase 2	↑1.94	toll receptor signalling	0.02
<i>SERPINE1</i>	serpin peptidase inhibitor, clade E	↓2.93	p53 signalling	0.00
<i>TICAM1</i>	toll-like receptor adaptor molecule 1	↑1.68	toll receptor signalling	0.04
			12 h PI	
<i>CASP8</i>	caspase 8, apoptosis-related cysteine peptidase	↑1.60	apoptosis signalling, cell-mediated immune response	0.04
<i>CDK1</i>	cyclin-dependent kinase 1	↑1.59	cell-mediated immune response, apoptosis signalling	0.00
<i>RRM2</i>	ribonucleotide reductase M2 polypeptide transcript variant 1	↑1.76	p53 signalling	0.00
<i>MSH2</i>	mutS homolog 2, colon cancer, nonpolyposis type 1	↑1.62	inflammatory response	0.00
<i>IL8</i>	interleukin 8	↑1.92	inflammatory response, immune cell trafficking, interleukin signalling pathway	0.00
<i>SREBF1</i>	sterol regulatory element binding transcriptio factor 1	↑1.67	inflammatory response, immune cell trafficking	0.03
<i>BRCA1</i>	breast cancer 1, early onset	↑1.60	cell-mediated immune response	0.00
<i>EGR1</i>	early growth response 1	↑1.62	cell-mediated immune response	0.00
			24 h PI	
<i>BOLA-DMB</i>	major histocompatibility complex, class II, DM beta-chain, expressed	↓3.29	T cell activation	0.02
<i>CASP8</i>	caspase 8, apoptosis-related cysteine peptidase	↑1.59	p53 signalling, inflammatory response, FAS signalling, apoptosis signalling	0.00
<i>CFB</i>	complement factor B	↓3.12	inflammatory response	0.01
<i>CXCL2</i>	chemokine (C-X-C motif) ligand 2	↑1.78	inflammatory response, immune cell trafficking	0.00
<i>DEFB103B</i>	defensin, beta 103B	↑1.88	inflammatory response	0.00
<i>EGR1</i>	early growth response 1	↑1.76	inflammatory response, immune cell trafficking, cell-mediated immune response, inflammatory response, immune cell trafficking, IL6 signalling, IL10 signalling, toll receptor signalling	0.00
<i>IL18</i>	interleukin 18 (interferon-gamma-inducing factor)	↑1.54	inflammatory response, immune cell trafficking, IL6 signalling, IL10 signalling, toll receptor signalling	0.00
<i>IL1F10</i>	interleukin-1 family member 10	↓5.26	IL6 signalling, IL10 signalling	0.00
<i>IL1RN</i>	interleukin 1 receptor antagonist	↑1.55	inflammatory response, immune cell trafficking, IL6 signalling, IL10 signalling	0.00
<i>IL8</i>	interleukin 8	↓2.76	inflammatory response, immune cell trafficking, IL6 signalling	0.00
<i>IRF3</i>	interferon regulatory factor 3	↑1.50	toll receptor signalling, signalling	0.00
<i>ITPR1</i>	inositol 1,4,5-triphosphate receptor, type 1	↓3.42	B cell activation, T cell activation	0.00
<i>LYZ1</i>	lysozyme 1	↓2.48	inflammatory response	0.00
<i>NOS2</i>	nitric oxide synthase 2, inducible	↓2.70	inflammatory response	0.00
<i>PTX3</i>	pentraxin 3, long	↓3.79	inflammatory response, immune cell trafficking	0.00
<i>S100A12</i>	S100 calcium binding protein A12 (calgranulin C)	↑1.87	inflammatory response	0.00
<i>S100A7</i>	S100 calcium binding protein A7	↓2.02	inflammatory response	0.00
<i>S100A8</i>	S100 calcium binding protein A8	↑1.82	inflammatory response, immune cell trafficking	0.00
<i>S100A9</i>	S100 calcium binding protein A9	↓2.21	inflammatory response, immune cell trafficking	0.00
<i>SERPINE1</i>	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	↑1.80	p53 signalling	0.00
<i>TGFB1</i>	transforming growth factor, beta 1	↑1.76	inflammatory response	0.00
<i>TLR2</i>	toll-like receptor 2	↑1.52	inflammatory response, toll receptor signalling	0.04
<i>TNFαIP3</i>	tumor necrosis factor, alpha-induced protein 3	↑1.81	inflammatory response	0.00

*The genes were manually selected from the list of differentially expressed genes, comprising the enriched immune-related pathways. Bold script denotes genes differentially expressed at multiple time-points post infection.

† fold change. Only genes with statistically significant fold changes (false discovery rate – FDR<0.05) were included in the table. Arrows in the column denote up-regulation (↑) or down-regulation (↓).

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