

## MYCOPLASMA AND HOST INTERACTION: *IN VITRO* GENE EXPRESSION MODULATION IN *MYCOPLASMA SYNOVIAE* AND INFECTED CHICKEN CHONDROCYTES

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The complex interplay between *Mycoplasma synoviae* and chicken chondrocytes (CCH), which come into direct contact during infectious synovitis, has been examined at the level of gene expression. Our previous studies demonstrated a significant influence of *M. synoviae* on the level of CCH gene expression. Here, we show for the first time that *in vitro* co-cultivation of *M. synoviae* and CCH also induces upregulation of gene expression in this mycoplasma. We observed significantly increased expression of genes important for *M. synoviae* pathogenicity, including cysteine protease *cysP*, neuraminidase *nanH*, haemagglutinin *vlhA*, and the putative nuclease MS53\_0284. Moreover, the pattern of gene expression was dependent on the infection environment. In CCH, significant changes in the expression of genes encoding catabolic enzymes of the cartilage extracellular matrix (cathepsins B, K and L, aggrecanase ADAM10, and matrix metalloproteinase MMP2) were demonstrated. Infection of CCH with *M. synoviae* also elevated the expression of the gene encoding peptidyl arginine deiminase, type III (PADI3), which is responsible for the post-translational citrullination of proteins.

**Key words:** *Mycoplasma synoviae*, chicken chondrocytes, gene expression, pathogenicity, enzymes

Mycoplasmas are the smallest self-replicating obligatory parasitic bacteria yet discovered, which have a specific cell membrane structure and exhibit an extreme reduction in genome size (Razin et al., 1998). Until recently, it was thought that mycoplasmas regulated gene expression independently of environmental signals, rather than sensing and responding to them, and that their major survival strategy depended on genetic mechanisms of random antigenic variation (Razin et al., 1998). However, Cecchini et al. (2007) showed that *Mycoplasma gallisepticum* responded to exposure to eukaryotic cells with transcriptional regulation, while Hallamaa et al. (2008) provided evidence of transcriptional regulation of

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*M. pneumoniae* lipoprotein genes in response to *in vitro* contact with human epithelial cells.

*Mycoplasma synoviae* colonises the upper respiratory tract of poultry where it causes subclinical infection, while the more invasive strains can cause systemic infection and arthritis (Lockaby et al., 1998; Narat et al., 1998). This phenotype of mycoplasma synthesises variable lipoprotein haemagglutinin VlhA, immunodominant variably expressed membrane lipoproteins that play an important role in binding to the host cell receptors, enabling the colonisation of host tissues. Experimental infection of chickens with *M. synoviae* has been found to thin the articular cartilage (Kerr and Olson, 1970) while Dušanić et al. (2012) showed that *M. synoviae* infection induces the expression of genes involved in apoptosis in chicken chondrocytes (CCH). In previous studies we identified two *M. synoviae* proteins that can modify chicken proteins: cysteine protease CysP, which cleaves chicken IgG into Fab and Fc fragments, and neuraminidase NanH, which desialylates chicken IgG and tracheal mucus glycoproteins *in vitro* (Berčič et al., 2011; Cizelj et al., 2011). The genome of *M. synoviae* contains two genes that encode nucleases (MS53\_0284 and MS53\_0110), although the respective proteins have yet to be reported (Vasconcelos et al., 2005). Meanwhile, it has recently been postulated that autoimmunity, cartilage degradation and chondrocyte degeneration are correlated with the presence of cathepsins B, K and L, aggrecanases and metalloproteinases (Goldring and Marcu, 2009).

The aim of the present study was to investigate whether environmental factors, such as host cell cytoplasm as well as surface and soluble factors from CCH, influence *M. synoviae* gene expression.

## Materials and methods

### *Cultivation of chicken chondrocytes*

Chicken chondrocytes (CCH) were isolated from hyaline cartilage as described previously (Dušanić et al., 2009) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 7.5% fetal bovine serum (FBS, Sigma-Aldrich) and 2.5% chicken serum (Sigma-Aldrich). Cells were cultured at 37 °C in an incubator with 5% CO<sub>2</sub> atmosphere. The chicken hyaline cartilage tissue was obtained from chickens in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA). The protocol was approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection (Permit Number: 34401-27/2012/11).

### *Cultivation of Mycoplasma synoviae*

Cultures of *M. synoviae* type strain WVU 1853 (Morrow et al., 1990) were cultured in modified Frey's medium at 38 °C temperature as described previously (Dušanić et al., 2009). Approximately 20 *in vitro* passages were performed prior to culture, for which *M. synoviae* in logarithmic growth phase was used for CCH infection. The number of colony forming units (CFU) was determined by the standard procedure (Rodwell and Whitcomb, 1983).

### *Experimental design*

CCH were seeded at  $5 \times 10^5$  cells per T25 vented tissue-culture flask (Greiner Bio One) and incubated overnight at 37 °C in 5% CO<sub>2</sub>. Based upon the results of our previous studies (Dušanić et al., 2009, 2012), *M. synoviae* broth culture (MS0) in logarithmic growth phase was used to infect CCH at a ratio of ~50 *M. synoviae* CFU per CCH. After 24, 48 and 72 h of incubation at 37 °C in 5% CO<sub>2</sub>, culture supernatants were collected and CCH were sampled by trypsinisation. After washing in sterile phosphate-buffered saline, supernates were combined with the corresponding culture supernatant. In this way two sets of samples were obtained: (i) a pellet containing CCH and *M. synoviae* attached to or invaded into CCH (MS-cch24, MS-cch48, MS-cch72), and (ii) supernatants with unattached *M. synoviae* (MS-sup24, MS-sup48 and MS-sup72), which were further centrifuged to obtain a pellet of bacteria (Table 1). As controls, *M. synoviae* broth culture used for infection of CCH (MS0) and CCH culture after 24, 48 or 72 h of incubation (CTRL24, CTRL48 and CTRL72) were used. Total RNA from all samples was isolated using RNeasy Mini Kit (Qiagen GmbH, Germany) and stored at -80 °C. RNA was purified from residual DNA with RNase-free DNase I (Fermentas, Germany) and 1 µg of total RNA was transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA), according to the manufacturer's instructions.

### *Gene expression analysis in M. synoviae*

RT-qPCR analysis was performed following a previously described protocol (Dušanić et al., 2012). Briefly, 20 µL mixtures were made, containing 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, USA), 5 µM of each primer (Integrated DNA Technologies, Belgium), 8 µL of DEPC-treated water (Qiagen GmbH, Germany), and 1 µL of cDNA. Stratagene Mx3000P (Agilent Technologies – Stratagene, USA) was used to perform RT-qPCR reactions and the manufacturer's MxPro software for analysis of amplification and dissociation plots. Gene expression values of MS broth culture (MS0) were used for gene expression calibration. Appropriate controls (no template and no reverse transcription control) were performed in each run. Due to the ability of *M. synoviae* to attach to and invade CCH (Dušanić et al., 2009), cDNA templates transcribed from

MS0, MS-sup24, MS-sup48 and MS-sup72 were used along with samples of infected CCH in which RNA was expected to be produced from *Mycoplasma* cells that have invaded or been attached to CCH cells (MS-cch24, MS-cch48, MS-cch72) (Table 1). Gene expression was tested for four genes (Table 2). The genes encoding proteins involved in *M. synoviae* pathogenicity were included in the gene expression analysis (Berčič et al., 2011; Cizelj et al., 2011). Genes *tufA*, *rsuA* and *gidB* were used as reference genes based on the stability of their expression during our studies. Prior to use, primer pairs were checked for specificity *in silico* using NCBI Primer BLAST. Primer efficiency was checked for each primer pair using dilutions of cDNA combined from all experimental conditions and time points.

#### *Gene expression analysis in M. synoviae-infected CCH*

RT-qPCR analysis was performed as described for gene expression analysis in *M. synoviae*. Briefly, gene expression was assayed for six genes (Table 3). The expression of genes RPL13, TBP and HPRT1 did not vary over the course of the experiment and therefore these genes were used as CCH reference genes. Gene expression values of non-infected CCH were used for gene expression calibration.

#### *Statistical analysis of gene expression data*

Three independent experiments were performed to collect RNA for RT-qPCR. Relative gene expression was assayed for each experiment and experimental condition separately. Three repeats of each RT-qPCR reaction were performed. Normalized relative quantities were obtained using the efficiency corrected  $2^{-\Delta\Delta C_q}$  method (Pfaffl, 2001). Statistical significance between samples from different experimental groups was determined using the unpaired Student's *t*-test ( $P < 0.05$ ).

## **Results**

#### *Infection of CCH-induced gene expression in M. synoviae*

Analysis of *M. synoviae* gene expression revealed three different profiles. The gene expression profile of attached and invaded *M. synoviae* (MS-cch) differed from that of *M. synoviae* collected from infected CCH culture supernatant (MS-sup), and from that of the control (MS0) (Fig. 1). The *M. synoviae* cells that infected CCH (attached on or invaded into CCH) reached a peak of expression of MSPA mRNA at 48 h after infection (MS-cch48) (11.9-fold,  $P < 0.0001$ ) and remained elevated after 72 h (MS-cch72) (4.4-fold,  $P < 0.0004$ ) compared to expression of MSPA mRNA in MS0. We observed a significant increase (5.6-fold;

**Table 1**  
Overview of experimental setup and sample names

| RNA sample                                                | Sample name       | Gene expression analysis of                                                        |
|-----------------------------------------------------------|-------------------|------------------------------------------------------------------------------------|
| <i>M. synoviae</i> culture used for infection             | MS0               | <i>M. synoviae</i> (control) <sup>1</sup>                                          |
| CCH infected with <i>M. synoviae</i> for 24, 48, and 72 h | MS-cch 24, 48, 72 | Attached and invaded <i>M. synoviae</i> <sup>1</sup> and infected CCH <sup>2</sup> |
| <i>M. synoviae</i> from supernatants of infected CCH      | MS-sup 24, 48, 72 | Unattached <i>M. synoviae</i> in CCH culture supernatant <sup>1</sup>              |
| Non-infected CCH incubated for 24, 48, and 72 h           | CTRL 24, 48, 72   | CCH (control) <sup>2</sup>                                                         |

<sup>1</sup>For gene expression, primers in Table 2 were used; <sup>2</sup>For gene expression, primers in Table 3 were used

**Table 2**

A list of oligonucleotides used as primers in RT-qPCR analysis of gene expression in *Mycoplasma synoviae*

| Gene symbol         | RefSeq GenBank acc. number | Encoding protein function                                      | Forward primer              | Reverse primer            |
|---------------------|----------------------------|----------------------------------------------------------------|-----------------------------|---------------------------|
| <i>cysP</i>         | YP_278708.1                | Cysteine protease                                              | AAGCATAAGCAAAATCCAACAG      | AACACATCTCCAATCTTCCAA     |
| <i>nanH</i>         | EU_245026                  | Neuraminidase (nanH)                                           | TTATCATCGTAGGTCATTCT        | CAACCAGTATTGAAAACCTTAGA   |
| <i>MSPA (vilhA)</i> | HQ326479.1                 | Variable lipoprotein haemagglutinin (C-terminal part of VilhA) | TCTGTGTCAGCTTCTTCTTACCT     | GCAGTACTCCAACAACAAGGCAA   |
| <i>MS33_0284</i>    | YP_278410.1                | Hypothetical protein (staphylococcal nuclease homologues)      | TGGTGAAGCCACTATTGAATCGCT    | GCAACAACAGCATCAAAGTATCCAC |
| <i>rsuA</i>         | YP_278540.1                | 16S rRNA uridine-516 pseudouridylylate synthase                | CCTTCGGTATCGGATATCTAGTCTCCG | AGGAATCTTCTCGAGGCCAGA     |
| <i>gidB</i>         | YP_278282.1                | 16S rRNA methyltransferase GidB                                | AGCCAACGCCGAGAACCGATATCTA   | CCTAACAGGATTTAGTGGCGAGTC  |
| <i>tufA (ef)</i>    | YP_278785.1                | Elongation factor Tu (MS53_0667)                               | TTCTTCGTCATCAACCCAT         | ACTCGTGAACACACATTCTT      |

**Table 3**  
A list of oligonucleotides used as primers in RT-qPCR analysis of gene expression in chicken chondrocytes

| Gene symbol   | RefSeq mRNA number | Encoding protein function                | Forward primer             | Reverse primer           |
|---------------|--------------------|------------------------------------------|----------------------------|--------------------------|
| <i>MMP2</i>   | NM_204420          | Matrix metalloproteinase 2               | TGGTGTGCTTCTACCAGCACGTAT   | AATTCTGGGCAGCAACCAAGAAG  |
| <i>PADI3</i>  | NM_205043          | Peptidyl arginine deiminase, type III    | AGCACCGAGGAAAGCATCTTCT     | CCAGCAGTGTGACATGGAATGCAA |
| <i>CTSL2</i>  | NM_001168009       | Cathepsin L2                             | GAAAGTCAGAAAGGAAAGTACAGAGG | CTCTCCAGTCAACAGATCGTG    |
| <i>CTSK</i>   | NM_204971          | Cathepsin K                              | ATTGGTGTACTGCGTCTCC        | CATACAGCTCTCATCCTGCC     |
| <i>CTSB</i>   | NM_204261          | Cathepsin B                              | TGGCTTCGAGTGGGGCATGG       | GGTGGCCGGGAGCCATTGAC     |
| <i>ADAM10</i> | NM_205371          | ADAM metalloproteinase domain 10         | GCCTCTCAGCCTAAACAGAAC      | CATCCTTGCCATCCGAACTAG    |
| <i>TBP</i>    | NM_205103          | TATA box binding protein                 | CCCCTTTGCCGGGAACCA         | TCGGGCACGAAAGTGCAATGG    |
| <i>HPRT1</i>  | NM_204848          | Hypoxanthine phosphoribosyltransferase 1 | GACGGGAAAGCAGAAGTACAAAGC   | ACGATGGGGCTGGGGTGTTT     |
| <i>RPL13</i>  | NM_204999          | Ribosomal protein L13                    | TGTCCGGACCGGTTATGCCGA      | AGAGACGAGCGGTTTGCCCCGG   |

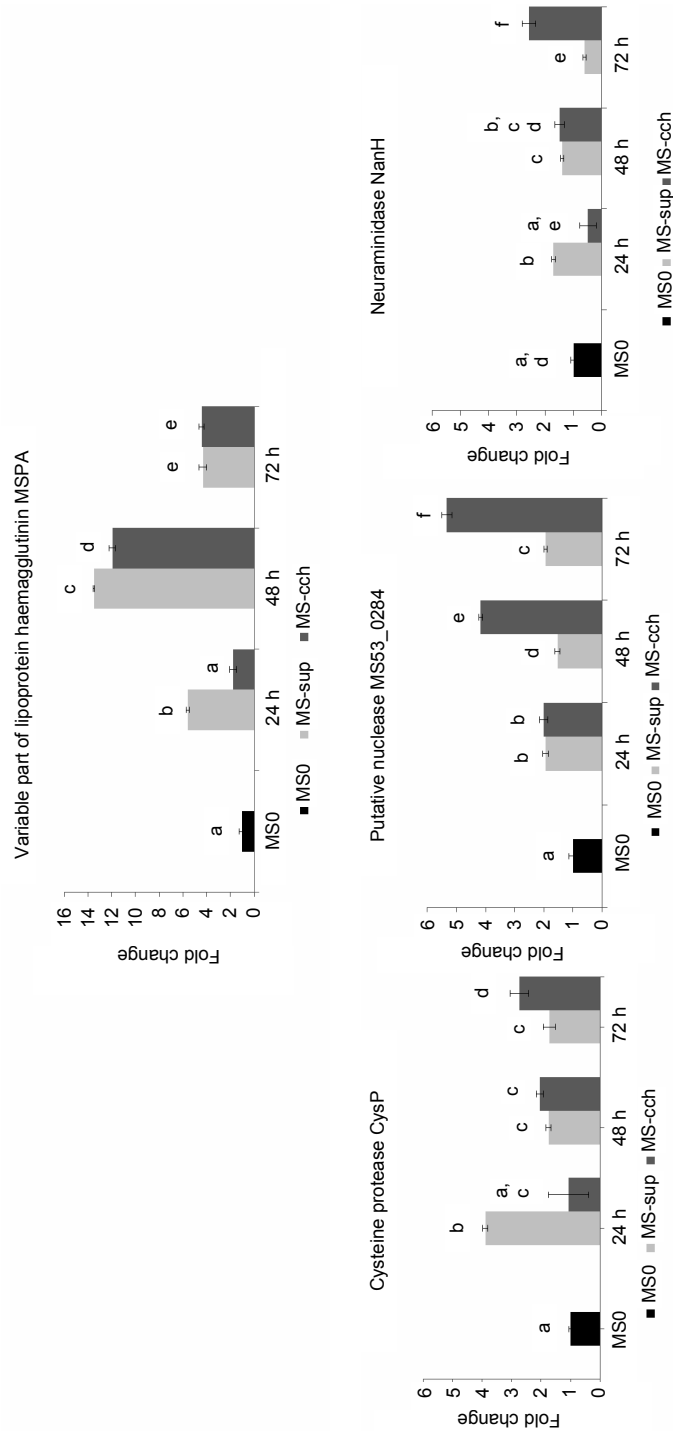


Fig. 1. Expression of selected *Mycoplasma synoviae* genes after incubation with CCH. Control (MS0) represents expression of a particular gene in *M. synoviae* culture that was used to infect CCH. The results are given as mean values  $\pm$  standard error for three independent experiments with three RT-qPCR replicates for each experiment. Means with a different letter are statistically different ( $P < 0.05$  to  $P < 0.001$ ) by Student's *t*-test

$P < 0.0001$ ) in expression of MSPA mRNA in *M. synoviae* from supernates after 24 h of incubation (MS-sup24), a 13.5-fold increase ( $P < 0.0001$ ) at 48 h post infection (PI) (MS-sup48) and a 4.3-fold increase ( $P < 0.002$ ) at 72 h PI (MS-sup72) compared to the *M. synoviae* culture used for infection of CCH (MS0) (Fig. 1).

The concentrations of CysP mRNA in *M. synoviae* cells that infected CCH were at the basal point at 24 h PI, but increased 2.7-fold during infection (MS-cch72,  $P < 0.006$ ) compared to the expression in MS0. Concentrations of CysP mRNA in *M. synoviae* CCH supernates were significantly increased (3.9-fold,  $P < 0.0001$ ) at 24 h PI compared with MS0, but returned to nearly basal concentrations at 48 h PI (1.7-fold,  $P < 0.003$ ), staying at the same level at 72 h (Fig. 1).

The expression of MS53\_0284 in *M. synoviae* CCH supernates was induced 1.9-fold at 24 h PI ( $P < 0.006$ ) compared that in MS0, and 2.0-fold ( $P < 0.005$ ) compared to that in infected CCH. The expression of MS53\_0284 mRNA in *M. synoviae* cells collected from supernates remained at the same level also after 72 h PI, while in *M. synoviae* cells that infected CCH the expression of MS53\_0284 mRNA increased after 48 h PI to 4.2-fold ( $P < 0.0001$ ), reaching a maximum at 72 h (5.3-fold,  $P < 0.0001$ ) (Fig. 1).

The expression of the *nanH* gene was highest in *M. synoviae* cells that infected CCH at 72 h PI (2.6-fold,  $P < 0.004$ ) and in *M. synoviae* cells from infected CCH supernates at 24 h PI (1.7-fold,  $P < 0.003$ ) compared to the expression in MS0. Concentrations of NanH mRNA in both types of *M. synoviae* cells were approximately 1.4-fold higher than in MS0 at 48 h. The suppression in *nanH* gene expression was detected in *M. synoviae* cells that infected CCH at 24 h PI (0.5-fold) and in *M. synoviae* cells from infected CCH supernates at 72 h PI (0.6-fold,  $P < 0.004$ ) (Fig. 1).

#### *Gene expression of CCH after infection with M. synoviae*

The expression of genes encoding several catabolic enzymes of the cartilage extracellular matrix (cathepsins B, K and L, aggrecanase ADAM10 and matrix metalloproteinase II, MMP2) were induced following 48 h infection of CCH with *M. synoviae*. Hence, the expression of MMP2 increased 3.4-fold ( $P < 0.02$ ), cathepsin L (CTSL2) 3.6-fold ( $P < 0.01$ ), cathepsin K (CTSK) 2.7-fold ( $P < 0.01$ ), cathepsin B (CTSB) 2.1-fold ( $P < 0.04$ ), and aggrecanase ADAM 10 (ADAM10) 6.0-fold ( $P < 0.01$ ). Interestingly, the expression was not significantly increased in CCH after 24 h and 72 h infection, while expression of cathepsin B was increased approximately 2-fold at all measurement times. In addition to genes whose encoding proteins presumably contribute to cartilage destruction, a gene encoding the enzyme peptidyl arginine deiminase III (PADI3) was analysed for changes in gene expression. Compared to the genes encoding catabolic enzymes, PADI3 showed a significantly stronger level of upregulation after 48 h of CCH infection with *M. synoviae* (16.7-fold,  $P < 0.01$ ) (Fig. 2).



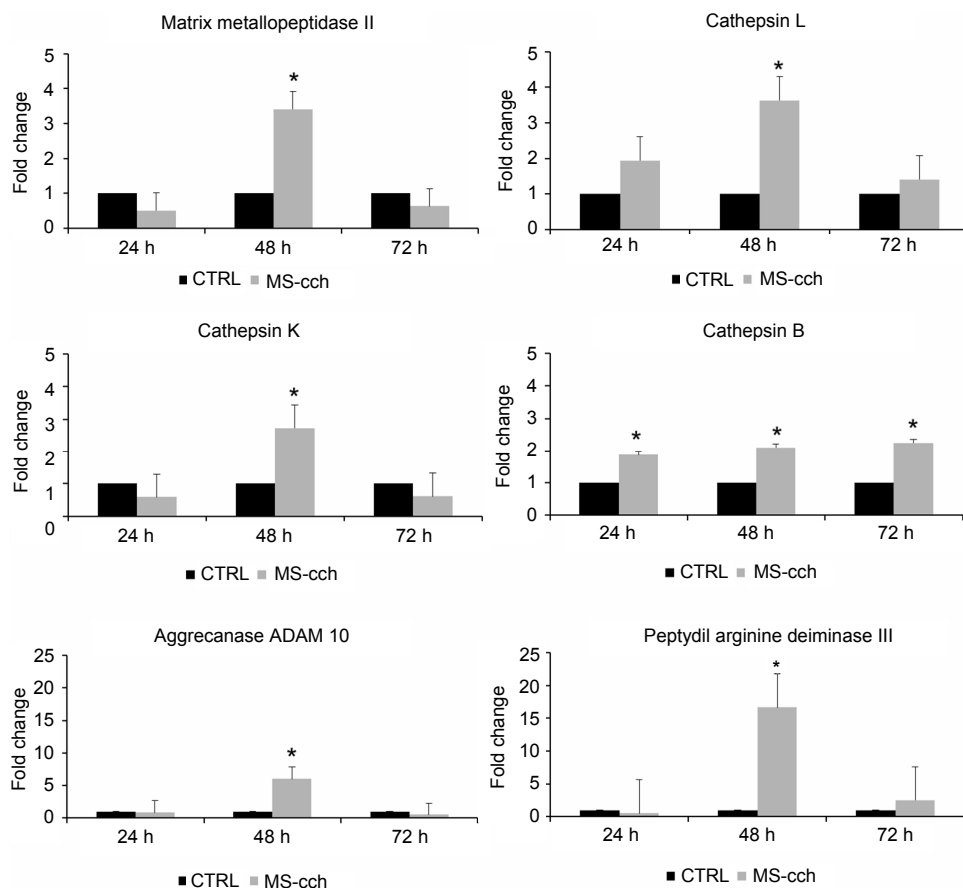


Fig. 2. Expression of selected genes in CCH after exposure to *Mycoplasma synoviae* WVU 1853. The control (CTRL) in all graphs represents non-exposed CCH. The results are given as mean values  $\pm$  standard error for three independent cell treatment experiments with three RT-qPCR replicates for each experiment. Means marked with stars are statistically different from controls ( $P < 0.05$  to  $P < 0.001$ ) by Student's *t*-test

## Discussion

To investigate *M. synoviae* induced by host cells, we exposed CCH to mycoplasma and explored the expression of several genes involved in *M. synoviae* pathogenicity and also genes encoding cartilage-degradation-linked enzymes in CCH. Mycoplasmas, including *M. synoviae*, are associated with joint infections leading to cartilage degradation and induction of autoimmunity. In addition to enzymes for degradation of extracellular matrix, PADI3 encodes an enzyme responsible for citrullination of proteins linked to the induction of autoimmunity in human rheumatoid arthritis (Yamada, 2005; Uysal et al., 2010).

Our previous studies found that 24 h after infection,  $1.2 \pm 0.3\%$  of *M. synoviae* invaded CCH, while other *M. synoviae* cells were attached to CCH or remained in culture supernates (Dušanić et al., 2009). Invaded and attached bacteria are in direct contact with CCH, the cytoplasm and surface of which can be considered as different infection environments than culture supernatants. Since all bacteria originated from a single broth culture, and RNA was isolated for all tested conditions at the same time, the expression of genes could be directly compared. Under two different environmental conditions, time-dependent trends of gene expression for CysP, NanH and MS53\_0284 proceeded in opposite directions, increasing in attached or invaded *M. synoviae* and decreasing in culture supernatant *M. synoviae*. The *vlhA* gene of *M. synoviae* yields a product that is cleaved post-translationally into an N-terminal lipoprotein (MSPB) and a C-terminal haemagglutinin protein (MSPA). Both MSPA and MSPB are surface-exposed proteins and exhibit high frequency antigenic variation, but only MSPA mediates binding to erythrocytes and is involved in the adhesion process (Noormohammadi et al., 1997). In our experiments for both environments the upregulation at all times with a peak at 48 h was observed for the gene encoding the most prominent adhesin in *M. synoviae*, the MSPA part of VlhA. Our results indicate that the host cell environment influences upregulation of the adhesion-associated gene in *M. synoviae*.

The highest gene expression of *cysP* and *nanH* in bacteria present in the culture supernatant was detected at 24 h PI. Although there is a link between VlhA and bacterial capacity to cause infectious synovitis (Narat et al., 1998), no such correlation has been observed for CysP so far. According to our observations, *M. synoviae* cleaves chicken IgGs, which are also found in CCH growth medium (Cizelj et al., 2011). Degradation of host IgG could enable *M. synoviae* to escape the immune system defence. This mechanism is more effective for non-attached and non-invaded bacteria, which is supported by our results.

Neuraminidase NanH of *M. synoviae* has been found to desialylate chicken IgGs and tracheal mucus glycoproteins (Berčič et al., 2011). Desialylation is important for tissue colonisation and could explain the high level of gene expression at 24 h PI. Both CysP and NanH could be important for providing *M. synoviae* with sources of energy and other growth requirements. Therefore, upregulation of both genes in attached and invaded bacteria at 72 h PI could be a response to needs for such sources.

The hypothetical protein MS53\_0284 of *M. synoviae* is noted as a staphylococcal nuclease homologue (Vasconcelos et al., 2005). Upregulation of MS53\_0284 in *M. synoviae* attached and invaded into CCH suggests that this putative nuclease may play a role in the host's nucleic acid degradation. This result may be relevant to our previous findings on *M. synoviae*-induced apoptosis in CCH (Dušanić et al., 2012). The upregulation of MS53\_0284 gene in mycoplasma cells that were in supernatants of infected CCH could be triggered by nucleic acids released from dead CCH cells.

In addition to the possible role of bacterial proteases in the cleavage of collagens, several proteases originating from immune cells, inflamed synovium and necrotic or apoptotic CCH have been implicated in cartilage degradation (Bartok and Firestein, 2010). Cartilage collagens and proteoglycans are a target of MMPs, aggrecanases and cathepsins B, K and L (Goldring and Marcu, 2009). The results of our study show that in CCH *M. synoviae* infection upregulates the expression of aggrecanase, metalloproteinase and cathepsins K and L at 48 h PI, while cathepsin B gene was upregulated at all times. This indicates that *M. synoviae* may play a role in tissue degradation in infectious synovitis by inducing gene expression in CCH. It has been reported that *M. hyorhinitis* and *M. arthritis* are able to cause a significant release of calcium from bone tissue (Novak et al., 1995). Increases in calcium concentration during inflammation could be a stimulus for activation of peptidyl arginine deimidase (PADI), leading to citrullination and the appearance of new epitopes for autoantibodies (Vossenaar et al., 2004). In the present study, PADI3 showed a significantly higher level of upregulation after 48 h of CCH infection with *M. synoviae*. These results could connect *M. synoviae* with the autoimmune nature of infectious synovitis.

In conclusion, the expression of genes important for the pathogenicity of *M. synoviae* is time dependent and influenced by environmental conditions. We have shown that *M. synoviae* induced the expression of genes encoding cathepsins B, K, L and PADI3 in CCH.

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