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MYCOBACTERIUM AVIUM SUBSP. *PARATUBERCULOSIS* AND THE EXPRESSION OF SELECTED VIRULENCE AND PATHOGENESIS GENES IN RESPONSE TO 6°C, 65°C AND PH 2.0

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ABSTRACT

The aim of this work was to study the expression of selected *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) genes connected with *MAP* virulence, adhesion and stress response. The temperature of 6°C and 65°C were chosen with regard to the food industry, storage conditions (refrigerator) and low-temperature pasteurization. A pH of 2.0, using lactic acid, was selected to mimic the natural environment of the stomach. Expression of selected genes was studied using real time reverse transcription PCR on three different *MAP* isolates. *MAP* isolates were chosen according to the number of their preceding cultivations. While isolates 8672 and 8819 were previously cultivated only once, *MAP* isolate 12146 went through four passages. Different expression profiles were observed in each of the three *MAP* isolates. However, particular similar patterns were observed. *SigE*, *sigF* and *ahpC* were up-regulated, while *sigL* was down-regulated under temperature stress. *Mmp* gene was found to be down-regulated under acidic conditions. Low passage isolates (8672 and 8819) showed certain level of acid resistance.

Key words: *MAP*; Johne's disease; stress; treatment; real time PCR

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is a slender, non-spore-forming, aerobic bacterium. Its main features are the requirement for Mycobactin in growth media, and slow growth, which complicates its cultivation and diagnosis. *MAP* is the causal agent of a chronic inflammation of the intestine called paratuberculosis or Johne´s disease (in Anglo-Saxon countries), which affects mainly domestic ruminants, but also other animal species. The disease is characterised by a loss of physical condition and weight, emaciation, decreased milk production and diarrhoea (3, 13, 18, 24,). Additionally, *MAP* is considered to be one of the possible causes of Crohn's disease (CD) in humans (7, 8, 26, 27, 37). The most frequent sources of *MAP* for humans are from contaminated drinking water, water aerosol, milk (14 – 16) or even meat (1).

Transcriptional control represents the major mechanism in gene expression regulation in prokaryotes and so the measurement of RNA transcripts provides an accurate picture

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of the cell's response to a given stress. The detection and quantification of gene expression levels can be investigated by many methods such as Northern blotting (2), S1 nuclease protection (6), sequencing of cDNA libraries (29) or serial analysis of gene expression - SAGE (43). Nowadays, real time reverse transcription qPCR (real time RT-qPCR) and microarray technology represent the most used techniques for expression profiling studies (45, 47).

The purpose of the present study was to investigate the *MAP* gene expression after inducing stress factors such as temperature and acid environment *in vitro*. The temperatures of 6°C and 65°C were chosen with regard to the storage conditions (refrigerator) and low-temperature pasteurization used in the food industry, respectively. A stress factor of pH 2.0 was selected with regard to the acid environment in the stomach. Appropriate candidate genes were selected for their involvement in the virulence, adhesion and stress response in *MAP* or other mycobacterial infections.

The most plentiful group was represented by sigma factors – transcriptional regulators, which are involved in all gene expression (23, 36, 44). Nineteen sigma factors are encoded by *MAP* genome (22). Mammalian cell entry (*mce*) genes represented the second largest group of tested genes; they were identified in many mycobacteria, and have been described to be important for pathogenesis (20 – 22, 31). *UmaA1*, *papA2*, *kdpC* and *impA* were investigated due to their role in various pathways contributing to colonisation of host tissues (38). The macrophage-induced gene (*mig*) and the major membrane protein gene (*mmp*) were selected because of their apparent involvement in virulence (4, 5, 11, 33, 34). *KatG* gene encodes the catalase-peroxidase enzyme contributing to mycobacterial survival in macrophages by protecting the cells against hydrogen peroxide (12, 19). In its absence, antioxidant protection is provided by an increased expression of *ahpC*, which is itself reduced by *ahpD* (30). Finally *gapdh* and *1g2* were selected as *MAP* housekeeping genes (12).

MATERIALS AND METHODS

Bacterial isolates

Three *MAP* cattle field isolates (12146, 8672 and 8819) from the Czech Republic were used. Isolates Nos. 8672 and 8819 were obtained from clinically manifested cows, while isolate No. 12146 originated from a healthy cow. Nevertheless, all isolates tested positive after cultivation on Middlebrook 7H9 with Middlebrook AODC enrichment, BD PANTA antibiotic mixture (all Becton Dickinson, Franklin Lakes, NJ, USA) and Mycobactin J (1 mg/l; Allied Monitor, Fayette, MO, USA). While isolates 8672 and 8819 went through preceding cultivation once, the third isolate, 12146, had been sub-cultivated four times (under the same conditions as previous two isolates). The *MAP* suspensions were inoculated into Middlebrook 7H9 broth supplemented with Mycobactin J (1 mg/l; Allied Monitor, USA) and OADC (Becton Dickinson, USA). Cells were incubated with shaking at 37°C for 5 to 10 weeks to log phase. All isolates were confirmed for the presence of IS*900* using real time qPCR (40; data not showed).

MAP treatments *in vitro*

Six ml of each *MAP* isolate in the log phase were transferred into glass tubes and subjected to 6°C, 65°C and lactic acid (LA) treatment. The tubes were exposed to 6°C and 65°C for 30 min. To ensure the acquirement of 65°C in the whole volume of *MAP* culture, tubes were exposed to "pre-heating" step (measured in the sealed glass tubes with 6 ml of *MAP* and containing a thermometer). The total time of 65°C temperature treatment, including the "pre-heating" step was determined as 31 min and 50 s. In the case of pH treatment, the appropriate concentration of LA (5%) was added into a tube containing 6 ml of *MAP* culture to obtain a pH of 2.0. The mixture was agitated for 2 minutes. A sample without any treatment was prepared to serve as a control. All experiments were carried out in two physical duplicates.

RNA isolation

Immediately after treatments, guanidine thiocyanate (4M, Sigma, St. Louis, MO, USA) was added to treated and untreated *MAP* cultures. The solution was allowed to stand at room temperature for 30 min prior to centrifugation (3,026 ×g for 10 min at 4°C). The pellet was resuspended in 600 µl of TriReagent (Sigma, USA) and homogenised with 0.1 mm Zirconia beads (BioSpec Inc., Bartlesville, OK, USA) using the MagnaLyser instrument (Roche Molecular Diagnostic, Penzberg, Germany) at 6,500 rpm for 15 s. The sample was homogenised a further three times with cooling on ice for 2 min between each step. Homogenised samples were transferred into a new tube, 100 µl of chloroform was added, gently mixed and centrifuged (10,000 ×g for 10 min at RT). The upper phase was transferred into a new tube and 350 µl of RLT buffer was added (RNeasy kit, Qiagen, Hilden, Germany). Subsequently, the RNeasy kit (Qiagen, Germany) was used for RNA isolation with these modifications: 250 µl of 98% ethanol was added to the mixture with RLT buffer and all centrifugation steps were carried out at 8,000 ×g for 20 s. RNA was eluted using 50 µl of RNase, DNase free water. The elution was repeated with the same filtrate again (RNA isolation according to Dr. Tim Bull, St. George's Hospital Medical School, London, UK; personal communication).

DNase treatment and reverse transcription (RT)

10 U of DNase I was applied to RNA samples according to the manufacturer's instructions (Roche Molecular Diagnostic, Germany). The reaction contained 40 µl of isolated RNA in a total volume of 50 µl. Incubation was carried out at 37°C for 30 min, followed by enzyme inactivation at 75°C for 5 min. After the first incubation, an additional 10 U of enzyme was added and the incubation and inactivation step was repeated. The RT mix contained 20 U of *Avian Myeloblastosis Virus*-reverse transcriptase (AMV-RT) and 1 × reaction buffer (Finnzyme, Espoo, Finland), 40 nmol of all dNTPs (Finnzyme, Finland), 40 U of Ribonuclease Inhibitor (Takara Bio Inc, Otsu, Japan), 1 pmol of each reverse primer (see Table 1), and 20 µl of DNase treated RNA. The mix was brought to a final volume of 40 µl using RNase free water. Reverse transcription was performed at 42°C for 60 min. After RT, cDNA was purified using the MinElute qPCR Purification Kit (Qiagen, Germany) with final elution into 100 µl of TE buffer (Amresco, Solon, OH, USA).

Real time qPCR

Real time qPCR mixes were made up to a final volume of 10 µl and contained 1 × of DyNAmo Probe qPCR Master Mix (Finnzyme, Finland), 0.1 U of Uracil DNA Glycosylase (Roche Molecular Diagnostic, Germany), 5 pmol of each forward and reverse primer, 0.5 pmol of each probe labelled with FAM and 1µl of appropriate cDNA. Primers and probes were designed using Primer3 software (35) on a *MAP* K10 strain genomic sequence (GenBank AE016958) and synthesised by VBC Genomics, Austria (Table 1). G*apdh* primers were described previously in Granger et al. (12). qPCR was performed using the LightCycler 480 Instrument (Roche Molecular Diagnostic, Germany) using 96-well qPCR plates under the following conditions: initial denaturation at 95°C for 15 min followed by 45 cycles of 95°C for 5 s and 60°C for 30 s (fluorescence data collection). Subsequent analysis was carried out using the "Second derivative maximum" option of the LightCycler 480 software (version 1.2.0.0625). All qPCR were done in triplicates.

Calculation of relative expression level

To determine the most suitable housekeeping genes for data normalization, the stability of genes was tested using geNorm software (42). Relative expression levels of selected genes were calculated according to the modified ΔΔCt method, which also takes into account real time qPCR efficiencies (32). Twice up- or down-regulated gene expression difference was considered to be significant (46, 48). The efficiency of real time qPCR for each set of primers and respective probes

(essential for the calculation of relative expression levels) was determined from serial dilutions of *MAP* isolate No. 12146 cDNA (1, 10, 100 and 1000 times diluted) in fish sperm DNA (Serva, Heidelberg, Germany). Real time PCR followed the above mentioned protocol.

Determination of *MAP* viability after the treatment

MAP viability was assessed by the propidium monoazide PMA-PCR (28). Briefly, from each culture (with treated or untreated control cells), two aliquots of 250 µl were delivered to individual tubes. One of them was subjected to PMA (Biotium, Inc, Hayward, CA, USA) treatment. Then, both tubes with PMA treated and untreated cells suspensions were centrifuged at 5,000 ×g for 5 min and the supernatant was removed. 250 µl of the fish sperm DNA (Serva, Germany) dissolved in TE buffer (50 ng/µl; Amresco, USA) was added to the pellet. The *MAP* cells were lysed at 100°C for 20 min, the suspension was then centrifuged and the supernatant served as a template for *F57* real time qPCR (40). Each sample was analysed in physical duplicate. For the determination of the *MAP* viability, the quotient of the absolute amount of *MAP* cells treated with PMA and without PMA was determined for respective *MAP* strain and treatment (control). By using this approach, we were able to determine the percentage of the *MAP* live cells, and to assess the effect of the treatment.

Table 1. Primers and probes used for the expression analysis in *Mycobacterium avium* subsp. *paratuberculosis*

Target	Name	Sequence	PCR product
1g2	1g2-F	aaacgatttgaacaaggtgctc	119 bp
	1g2-R	cgaatagggcgctgaatg	
	1g2-TM	FAM-atggaaggccacgaggcggatt-BHQ	
gapdh	GAPDH-F[a]	atcgggcgcaacttctacc	123bp
	GAPDH-R[a]	gtcgaatttcagcaggtgagc	
	GAPDH-TM	FAM-acgacatcaccgacaacagcacc-BHQ	
mmp	35kDa-F	cggagcagacgatccaga	82 bp
	35kDa-R	ggcgtcttccacaccttg	
	35kDa-TM	FAM-acgacctcgacgcgctgatc-BHQ	
fap-p	mod-F	gcatcaaccaggacagcac	83bp
	mod-R	gtcgctgaatttcacctcgta	
	mod-TM	FAM-ctcaacggcgccaacggaag-BHQ	
umaA1	umaA1-F	ttgacctacacccagaagcag	66bp
	umaA1-R	gaaccgtaaatcgctcatcg	
	umaA1-TM	FAM-cagcacgagcgcggcgtt-BHQ	
papA2	papA2-F	ggcgttcccacagaatcc	73bp
	papA2-R	cagacacatcgccctgac	
	papA2-TM	FAM-cgattcggtcgagcgctacatc-BHQ	
kdpC	kdpC-F	caccgttcgtgagcctct	76bp
	kdpC-R	atctggccgagcgaatagt	
	kdpC-TM	FAM-cgcgtcgaatgcgccaaga-BHQ	
impA	impA-F	ctgacctggttgccgttc	76 bp
	impA-R	gcgggatttcgttcttgc	
	impA-TM	FAM-ctcgaccagcgctacaccgc-BHQ	

	mce2-F	atccgcgctatgtcaacc	
mce2	mce2-R	cgtacttgttgccgaacagc	78 bp
	mce2-TM	FAM-tgattccggcgaacgtggtg-BHQ	
	mce3-F	caacacatcctgtcgattctc	
mce3	mce3-R	tggttgtcggtgatggtg	62 bp
	mce3-TM	FAM-tcggcgagcaccaccagc-BHQ	
	mce4-F	gacgctgggcatcaacag	
mce4	mce4-R	gccgaagatggtgttaccg	82 bp
	mce4-TM	FAM-tccaacgccaccgtgcacatc-BHQ	
	mig-F	ggccatatcgagctgctg	
mig	mig-R	cgtctcgacctcctcgac	81 bp
	mig-TM	FAM-actccgtgtgcatcaattccgg-BHQ	
	sigA-F	gatggcgttcctcgatttg	
sigA	sigA-R	agcccttggtgtagtcgaac	80bp
	sigA-TM	FAM-cctgatccgtgcggtcgagaa-BHQ	
	sigD-F	ccgtcgatgacaattccag	
sigD	sigD-R	ggagtgcgttgtggtctcc	79 bp
	sigD-TM	FAM-aacgtctcgatgctgtggtcgc-BHQ	
	sigE-F	gtgtaccggctggcctac	
sigE	sigE-R	ccggatgaaggtctcctg	75 bp
	sigE-TM	FAM-aatcagcacgacgccgaggac-BHQ	
	sigF1-F	gacgcagagccagatagcc	
sigF1	sigF1-R	agggtgtttgccaggatg	72 bp
	sigF1-TM	FAM-cgtctcgcagatgcaggtctcg-BHQ	
	sigF2-F	gcagctcctacaacaccttg	
sigF2	sigF2-R	cgcacttcctcctcttcg	61 bp
	sigF2-TM	FAM-tccatcgacagcggcggc-BHQ	
	sigH-F	gaccaacacctacatcaacagc	
sigH	sigH-R	gatttcctcggtcggatactc	70 bp
	sigH-TM	FAM-taccgcaagaagcagcgccag-BHQ	
	sigL-F	cgtgatcgaacggtcctact	
sigL	sigL-R	cgatgccgaggtctgtagc	65 bp
	sigL-TM	FAM-cggttggaccaccgcgcagata-BHQ	
	katG-F	caaccagggcaagttcgtc	
katG	katG-R	aagcggtcgttgttcatcac	66 bp
	katG-TM	FAM-aggacttcgtcgcggcctg-BHQ	
	ahpC-F	ctgaagaacctgccgttcc	
ahpC	ahpC-R	cgtcggcgttgagaacac	79 bp
	ahpC-TM	FAM-ctctcggacatcaagcgcgaact-BHQ	
	ahpD-F	gaacatcatcggcaatcc	
ahpD	ahpD-R	gaaacggcgaagcaccac	63 bp
	ahpD-TM	FAM-cgtggagaaggcgaacttcgagct-BHQ	

[a] Primers were taken from Granger *et al.* 2004

RESULTS

The testing of stability using geNorm software showed different stabilities of *1g2* and *gapdh* genes in different *MAP* isolates under various stress conditions (Fig. 1). As no other genes (from the set of 22) were found be suitable for use as a housekeeping gene for all three isolates, the geometric mean (GM) of all Cts (cycle thresholds) within the respective treatment (control) for each strain was calculated and used as a "normalization reference". Normalization using the GM has been described for larger set of data and represents a way of obtaining accurate expression profiles of tested genes (42). The relative expression levels for each *MAP* isolate are shown in Fig. 2. A two-fold change in expression was used as mentioned above.

MAP isolate 8672 showed seven up-regulated genes under temperature treatments 6°C and 65°C, which was the highest number of up-regulated genes from all tested isolates (Fig. 2). Isolate 8819 responded with 5 up-regulations, while only two up-regulations were observed in isolate 12146. Expression profiles were different in different isolates, nevertheless, a similar pattern was found. *SigE* was up-regulated in both 8672 and 8819 treated at 65°C. The same situation was noted with the gene *sigF2* under 6°C, although the expression in isolate 8819 was slightly under the limit of two. Similarly, *ahpC* was up-regulated in 8672, and was slightly below two in the isolate 12146 under the stress of 65°C (Fig. 2). *SigL* was the only common gene found to be down-regulated in two isolates (8819 and 8672).

With regards to the results of acidic treatments, the reverse was determined when comparing temperature stresses (Fig. 2). *MAP* isolate 12146 showed the highest level of response to this stress factor. Three genes (*gaphd*, *papA2* and *sigD*,) were up-regulated in isolate 12146 when compared to one *ahpC*, and no up-regulated gene in isolates 8672 and 8819, respectively. Each 8819 and 12146 showed down-regulation in two cases (*mmp*, *mig and impA* genes), whereas one down-regulated gene was noted in isolate 8672 (*mce2*). In both 12146 and 8819 isolates, the *mmp* gene was down-regulated. The correlation between stress factors and cell viability is shown in Table 2.

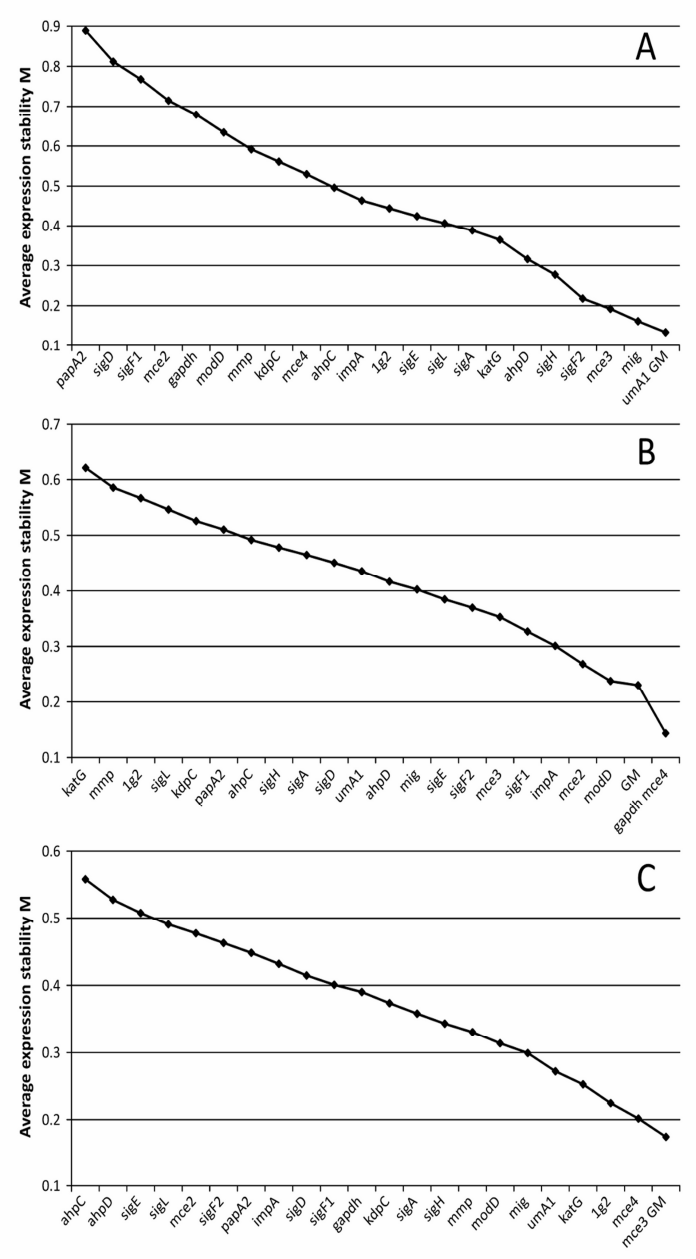

Figure 1. The average expression stability (M) of selected genes for the *Mycobacterium avium* subsp. *paratuberculosis* strains 12146 (A), 8819 (B) and 8672 (C) measured by GeNorm software. The lower M value the more stable gene.

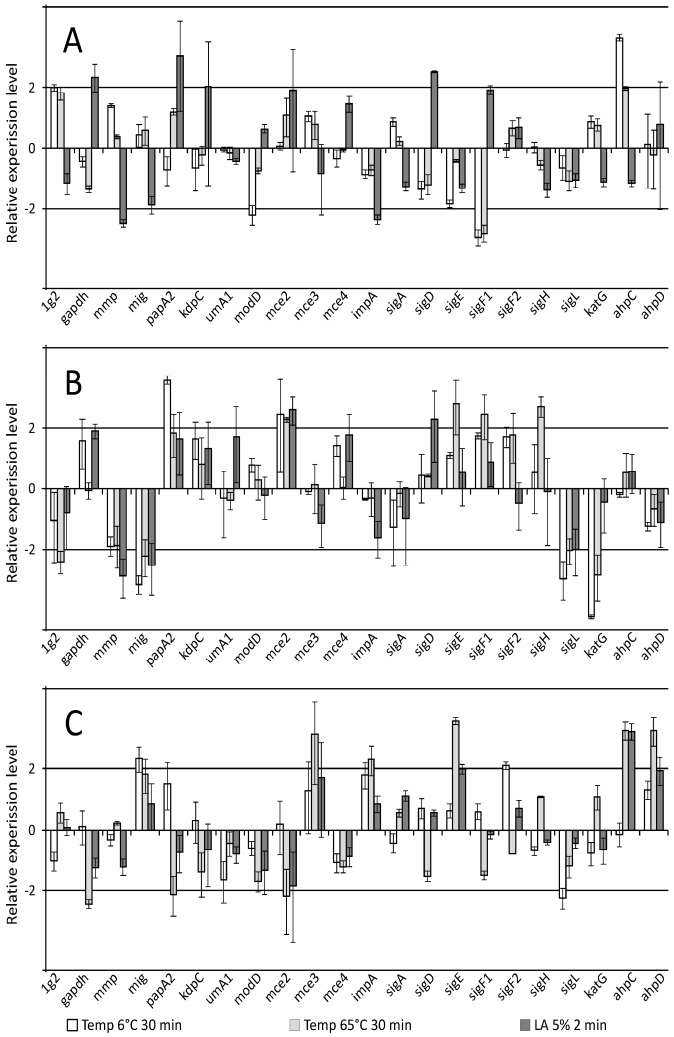

Figure 2. The relative gene expression levels (in log scale) of *Mycobacterium avium* subsp. *paratuberculosis* strains 12146 (A), 8819 (B) and 8672 (C). Numbers on y axis represent multiples of up- (positive values) and down- (negative values) regulation.

Table 2. Percentage of viable *Mycobacterium avium* subsp. *paratuberculosis* cells after treatments as determined by *F57* real time qPCR

	***MAP* 12146**	***MAP* 8672**	***MAP* 8819**
6°C	100.5	104.3	102.8
65°C	21.17	12.86	14.24
pH2	25.33	32.25	36.95

All calculations were done as the quotient of *MAP* cells treated with PMA and without PMA for the respective *MAP* strain.

DISCUSSION

The expression of virulence determinants in bacteria can be controlled by environmental signals, especially by temperature and low iron concentration, but osmolarity, pH, oxygen or CO_2 can play an important role as well (25). The goal of this study was to determine the influence of certain stress factors used in the food industry (storage conditions, low-temperature pasteurization and the presence of lactic acid) on the expression profiles of representatively selected genes involved in virulence, pathogenesis and stress response of *MAP*. A thorough examination of internet databases was carried out and suitable candidate genes were selected. The complete package contained 22 tested genes; two of them assumed to be housekeeping genes.

Two genes, *1g2* and *gapdh*, were selected to serve as housekeeping genes according to the literature (12). As they were previously described to be constitutively expressed in *MAP*, it was not confirmed in our study. None of the other genes were proven to be uniform for all three *MAP* strains therefore the strategy using a GM had to be adopted for normalization (42). This strategy is based on the principle that the mean expression level of a sufficiently large pool of genes is similar in all samples, regardless of the experimental conditions. Changes in the expression of a small portion of genes due to experimental conditions cannot change the overall mean of the expression ratio (49). For every control gene, the pairwise variation with all other control genes is determined as the standard deviation, and the internal control gene stability measure (M) is defined (42). Using the geNorm software, the GM was determined as the most stable (in 12146 and 8672 isolate) and second most stable (in 8819 isolate) "gene" (Fig. 1). We are aware that this study is the first to combine the use of GM and real time RT-qPCR for the determination of the reference gene. Although it is rather employed in the analysis of larger gene sets, we think that it could be utilised in real time RT-qPCR, especially when no suitable housekeeping genes are available.

Expression of the *mmp* gene was suppressed in both isolates 12146 and 8819 as a reaction to a low pH. In 8672 isolate *mmp* expression did not differ from the control sample. The *mmp* gene encodes a 35kDa major membrane protein (4, 5). Its transcription increases under high osmolarity and low oxygen tension, while no transcription occurs when exposed to acidic pH or standard aerobic growth conditions (5). Generally, 8819 isolate did not response at all under pH stress, while 12146 showed the highest number of up-regulated genes.

In all three *MAP* isolates, different genes were down-regulated under temperature stress. The transcription of *sigL* was suppressed under 6°C in both 8672 and 8819. *SigL* belongs to a family of transcriptional regulators. Hahn et al. (17) suggested *sigL* regulates the synthesis of cell envelope lipids and is responsible for the modification of secreted proteins in *M. tuberculosis*. They also found, *sigL* does not play a role in responding to oxidative stress. In *MAP*, two (8672 and 8819; Fig. 2) of three isolates showed decreased expression of *sigL* after cold shock. Also, it seems, *sigL* is not affected in by heat shock (45°C), acidic pH, and hyperosmolarity in *M. tuberculosis* (9). According to this study, higher temperature did not lead to changes in *sigL* expression in any of *MAP* isolates (Fig. 2).

45°C heat shock led to lower expression of *sigF* in a *M. smegmatis* study using the β-galactosidase enzyme as a reporter gene, whereas cold shock (15°C) led to higher expression of *sigF* when compared to the control (39). In *M. tuberculosis*, Manganelli et al. (23) observed no variation in the transcriptional level of *sigF* after cold shock, which is in contrast with the study of DeMaio et al. (10) who found up-regulation of *sigF* after cold shock in *M. bovis* BCG. In this study with *MAP* isolates, it seems, cold temperature leads to up-regulation of *sigF* (*MAP* isolates 8672 and 8819; Fig. 2) rather than to its down-regulation.

Under high temperature stress (65°C), the increased transcription of genes *sigF* and *sigE* was observed in isolates 8672 and 8819. The up-regulation of *sigE* has been observed after heat shock previously (23, 36). Wu et al. (46) describe the role of *sigE* in *MAP* virulence and pathogenesis, as this gene was co-regulated with a large number of other highly regulated genes. At 65°C, the transcription of *ahpC* and *ahpD* was also increased in isolate 8672. In isolate 12146, expression of *ahpC* was very slightly below the level of a two-fold change expression (1.95; Fig. 2). *AhpC* and *ahpD* genes encode detoxifying enzymes used for the protection against reactive nitric and oxidative metabolites. Olsen et al. (30), nevertheless, described a strong expression of the *ahp* genes *in vitro* without the bacteria being exposed to any oxidative stress.

The expression profiles of all three tested strains were different. The high passage isolate No. 12146 responded mostly to the acid treatment, when compared to the other two low passage *MAP* isolates. On the contrary, a relatively low change in 12146 gene expression during temperature stress was observed (Fig. 2). Sung and Collins (41) observed that a higher proportion of low passage *MAP* clinical isolates were sensitive to heat when compared to high passage strains. Thus, the temperature resistance of 12146 isolate (Tab. 2) rather reflects higher number of its previous passages than its real feature. On the contrary, isolate No. 8672 and 8819 mostly responded to temperature stress, while only a limited response to lactic acid stress was noticed (Fig. 2). As both 8672 and 8819 isolates went through preceding cultivation only once, a weaker reaction to acidic stress (Tab. 2, Fig. 2) could indicate a real level of their acid tolerance. As both these isolates originated from animals with manifested paratuberculosis, this feature could correspond with their pathogenesis and higher level of virulence.

This paper investigates the effect of temperature and pH stress on the expression of selected *MAP* genes connected to virulence and pathogenesis. Using three *MAP* isolates, various levels of gene expression for different genes were obtained, but certain similar patterns in expression were observed. Generally, the transcription of *sigF* was up-regulated under 6°C, while the transcription of *sigL* was down-regulated. Under higher

temperature stress (65°C), the expression of *sigE* and *ahpC* were found to be over-expressed comparing to control samples. Under acidic conditions, the expression of gene encoding the major membrane protein (*mmp*) was found to be decreased. Dissimilarities in gene expression in various *MAP* isolates most probably corresponded with the number of previous cultivations. Nevertheless, certain level of acid resistance was shown in low passage 8672 and 8819 isolates.

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