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.
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 (mmm) 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 subcultivated 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 IS900 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 x 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 x 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 1 µl 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). Gapdh 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 △△Ct 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.

| Target | Name | Sequence | PCR product |
|--------|------|----------|-------------|
| lg2    | 1g2-F | aaacgattgaacaaggtgctc | 119 bp |
|        | 1g2-R | cgataaggggegctgaatg | |
|        | 1g2-TM| FAM-atggaaggccagagggatt-BHQ | |
| gapdh  | GAPDH-F | atcgggcgaacctctacc | 123bp |
|        | GAPDH-R | gtgaatttcacaggtagc | |
|        | GAPDH-TM | FAM-acgacatcagcacaagc-BHQ | |
| mmp    | 35kDa-F | cgagacagagtccaga | 82 bp |
|        | 35kDa-R | ggcgcttcaaccttg | |
|        | 35kDa-TM | FAM-acgacactgcagctgtac-BHQ | |
| fap-p  | mod-F | gctacaaaagagaacag | 83bp |
|        | mod-R | gtcgtaattttactctga | |
|        | mod-TM | FAM-ctcaacggcgaacctg-BHQ | |
| umaA1  | umaA1-F | tgcacactcccaagacag | 66bp |
|        | umaA1-R | gaacgtaaatctctctga | |
|        | umaA1-TM | FAM-cgcacaggegctgt-BHQ | |
| papA2  | papA2-F | ggcgttcccaagatcc | 73bp |
|        | papA2-R | cgacacatcgcctgac | |
|        | papA2-TM | FAM-egattcgtccagctacat-BHQ | |
| kdpC   | kdpC-F | cgccgtgttcacctct | 76bp |
|        | kdpC-R | atctggccagagcaatgt | |
|        | kdpC-TM | FAM-egctgcaatgctgccag-BHQ | |
| impA   | impA-F | cgccgtgttctcttcg | 76 bp |
|        | impA-R | ggcggattgtcttcg | |
|        | impA-TM | FAM-ctcgcacagcctacccgc-BHQ |
| Gene | Forward Primer | Reverse Primer | TaqMan Probe | Length |
|------|----------------|----------------|--------------|--------|
| mce2 | atcgcgtatgccaac<br> cgtactggtgccgaacag<br> FAM-tgattccgggaacggttg-BHQ | 78 bp |
| mce3 | ccaacacacgctctgaga<br> tgtggtgctgtaggt<br> FAM-tcgccgagacccacagc-BHQ | 62 bp |
| mce4 | gacgtggtgccaacag<br> gccaagatgtgttaacc<br> FAM-tccaagccacgctgacct-BHQ | 82 bp |
| mig  | ggcctatgagcgtgta<br> cgtcctcgactcctcg<br> FAM-actccggtgctcaatccgg-BHQ | 81 bp |
| sigA | gatgccgttctcagattg<br> agcccttgggtgtagtcga<br> FAM-ctgtgatagcgcgaa-BHQ | 80 bp |
| sigD | cccctcgatagactcctc<br> gaaagctggttgcctc<br> FAM-aacgctgctcctgctc-BHQ | 79 bp |
| sigE | gatgaccggtcgtglytct<br> cccgtagaaggttctc<br> FAM-aagcagcagcagcagagc-BHQ | 75 bp |
| sigF1| gcacggtcataaaccctgtg<br> agggctgccggaaggt<br> FAM-tccgatccgataggacggtc-BHQ | 72 bp |
| sigF2| gcacggtcataaaccctgtg<br> agggctgccggaaggt<br> FAM-tccgatccgataggacggtc-BHQ | 61 bp |
| sigH | gacacacacgataaaccctgtg<br> cggactcactcctccta<br> FAM-tacgcaagacgagcagc-BHQ | 70 bp |
| sigL | cgtatcgggagttctcagt<br> cggcggcggcggcggc<br> FAM-tccgatccgataggacggtc-BHQ | 65 bp |
| katG | caacgagggcaagttcctc<br> aagcggctggttgctcatc<br> FAM-agggactgctggttgctcatc-BHQ | 66 bp |
| ahpC | ctgaagaacctgcggttc<br> cgtggtgctggaagcaac<br> FAM-tccgatccgataggacggtc-BHQ | 79 bp |
| ahpD | cgcagtcagcgaatcag<br> gaaacgggacaggacac<br> FAM-tccgatccgataggacggtc-BHQ | 63 bp |

*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 (gapdh, 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.
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\textsubscript{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 \textit{mmp} gene was suppressed in both isolates 12146 and 8819 as a reaction to a low pH. In 8672 isolate \textit{mmp} expression did not differ from the control sample. The \textit{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 \textit{MAP} isolates, different genes were down-regulated under temperature stress. The transcription of \textit{sigL} was suppressed under 6°C in both 8672 and 8819. \textit{SigL} belongs to a family of transcriptional regulators. Hahn et al. (17) suggested \textit{sigL} regulates the synthesis of cell envelope lipids and is responsible for the modification of secreted proteins in \textit{M. tuberculosis}. They also found, \textit{sigL} does not play a role in responding to oxidative stress. In \textit{MAP}, two (8672 and 8819; Fig. 2) of three isolates showed decreased expression of \textit{sigL} after cold shock. Also, it seems, \textit{sigL} is not affected in by heat shock (45°C), acidic pH, and hyperosmolarity in \textit{M. tuberculosis} (9). According to this study, higher temperature did not lead to changes in \textit{sigL} expression in any of \textit{MAP} isolates (Fig. 2).

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

Under high temperature stress (65°C), the increased transcription of genes \textit{sigF} and \textit{sigE} was observed in isolates 8672 and 8819. The up-regulation of \textit{sigE} has been observed after heat shock previously (23, 36). Wu et al. (46) describe the role of \textit{sigE} in \textit{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 \textit{ahpC} and \textit{ahpD} was also increased in isolate 8672. In isolate 12146, expression of \textit{ahpC} was very slightly below the level of a two-fold change expression (1.95; Fig. 2). \textit{AhpC} and \textit{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 \textit{ahp} genes \textit{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 \textit{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 \textit{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 \textit{MAP} genes connected to virulence and pathogenesis. Using three \textit{MAP} isolates, various levels of gene expression for different genes were obtained, but certain similar patterns in expression were observed. Generally, the transcription of \textit{sigF} was up-regulated under 6°C, while the transcription of \textit{sigL} was down-regulated. Under higher
temperature stress (65°C), the expression of \( \text{sigE} \) and \( \text{ahpC} \) were found to be over-expressed comparing to control samples. Under acidic conditions, the expression of gene encoding the major membrane protein (\( \text{mmp} \)) was found to be decreased. Dissimilarities in gene expression in various \( \text{MAP} \) 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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