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Research Article

Induction of DnaK and GroEL in *Brucella Ovis* under Various Stress Conditions

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Abstract

Brucella ovis is a facultative intracellular pathogen, able to survive and replicate within macrophages. The role of molecular chaperones in *Brucella* intracellular survival has been previously mentioned, but nothing was published on *B. ovis* in this respect, as far as we know. In the present work, in order to understand any possible contribution of the molecular chaperones DnaK and GroEL for *Brucella ovis* stress survival and intracellular persistence, the expression on different culture media under different stress conditions were analysed at the transcriptional and translational levels and compared with that of bacteria grown inside murine J774A.1 cells. The transcription analyses showed different levels of *dnaK* and *groEL* induction under various stress conditions (e.g., macrophages survival, acidic pH

shock, high temperature, oxidative and osmotic shock) and a significant influence of the media composition. These results were consistent with the level of DnaK and GroEL expression analysed by the traditional proteomic methods. Here we demonstrate the molecular chaperones involvement in macrophage-*B. ovis* interaction, their expression under stress conditions suggesting the chaperones role in the bacterial resistance to such unfavourable environmental conditions.

Keywords: Gene expression · *Brucella ovis* · DnaK · GroEL

Introduction

Brucella ovis is a facultative intracellular pathogen associated with epididymitis and abortion in sheep, being less studied than the zoonotic species of *Brucella*, although the infection produced by these bacteria represents an important economical problem in many countries. Survival and multiplication in host phagocytic cells are crucial in the pathogenesis of *Brucella* infections (Young, 2009; von Bargaen et al., 2012). Along with other adaptive mechanisms used by *B. ovis* (Celli, 2006; Guerra, 2007; Roop et al., 2009), a stress response would presumably increase the chances of pathogen survival and dissemination. The molecular chaperones DnaK (70 kDa) and GroEL (60 kDa) are highly immunogenic or stress proteins induced under various stress conditions in *Brucella melitensis*, *B. abortus* and *B. suis* (Hendrick

and Hartl, 1993; Lin and Ficht, 1995a; Köhler et al., 1996; Rafie-Kolpin et al., 1996; Teixeira-Gomes et al., 2000; Kohler et al., 2002). In other bacteria (*Escherichia coli*, *Lactobacillus rhamnosus* and *Caulobacter crescentus*), it has been reported by previous papers that GroEL and DnaK are induced during osmotic stress (Meury and Kohiyama, 1991; Bianchi and Baneyx, 1999; Prasad et al., 2003; Susin et al., 2006).

In *B. ovis*, DnaK and GroEL have been identified as immunogenic proteins (Teixeira-Gomes et al., 1997), but the involvement of these proteins in the resistance of *B. ovis* cells in macrophages are not mentioned in the literature, as far as we know.

In this study, the chaperone proteins expression of *B. ovis* grown inside murine J774A.1 cells has been compared with that of

bacteria grown in the different cell culture media under various stress conditions, such as acidic pH, high temperature and oxidative stress, which reproduce macrophage intracellular environment. We analyzed also the importance of DnaK and GroEL for *B. ovis* cells survival in the presence of high concentration of glucose.

A sensitive and powerful technique for quantification of nucleic acids, real time quantitative RT-PCR, was used for the estimation of the transcription of *dnaK* and *groEL* genes. In addition, the DnaK and GroEL expression was also analyzed at the translational level by SDS-PAGE and Western blot analysis.

Materials and Methods

Bacterial Strain and Culture Conditions

B. ovis 202 was obtained from the Bacteriology Department, IDAH (Institute for Diagnosis and Animal Health), Romania. This strain was confirmed as *B. ovis* by standard biotyping procedures described by Alton et al. (1988). *B. ovis* cells were grown in an atmosphere containing 5% CO₂ for 72 to 89 h at 37°C in three different culture media: tryptic soy broth (TSB) (Oxoid, Cat. No. CM0129B); brain heart infusion (BHI) broth (Oxoid, Cat. No. CM1135B) and Columbia agar base (Oxoid, Cat. No. CM0331B) with 5% sterile defibrinated blood. For all the experiments, in parallel with untreated culture (normal conditions), mentioned above, the stress conditions were induced in the three culture

media. For the heat shock response, the temperature was raised to 42°C (Lin et al., 1992). For the reduced pH (5.5), the protocol described by Rafie-Kolpin et al. (1996) was followed. The oxidative stress was induced by supplementing the medium with 50 mM H₂O₂ (Teixeira-Gomes et al., 2000). The response to osmotic stress was tested with *B. ovis* cultures in BHI broth by adding 5% glucose; *B. ovis* grown in TSB in normal conditions was used as positive control.

SDS-PAGE Analysis

Proteins were extracted with BioRad ReadyPrep Protein Extraction kit. Essentially, the wet cells (50 µl containing 50 µg of protein) were mixed with 1 ml of complete 2-D Rehydration/Sample Buffer 1, lysed with Greenbeads (Roche),

and centrifuged. The supernatant was mixed with sample buffer and incubated for 5 min at 95°C. Triplicate of identical protein concentration, as well as Broad Range molecular weight standard (Bio-Rad) were applied on polyacrylamide (PAA) gel. Gels were stained with 0.1% Coomassie Brilliant Blue R-250, destained by a solution containing methanol, acetic acid and water (4:1:5) and photographed dried.

Western Blotting (Immunoblotting)

After electrophoresis, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane using Semi-Dry Transfer Cell (BioRad) according to the instructions recommended by the manufacturer. The mouse anti-GroEL monoclonal antibodies, and the mouse anti-DnaK monoclonal antibodies were purchased

from StressGen Biotechnologies Corporation. Detection of stress proteins was done with Immun-Blot Assay kit (BioRad).

RNA Extraction and Reverse Transcription (RT)

RNA was stabilized in RNAprotect Bacteria Reagent (Qiagen). Extraction of total RNA from both the bacterial cultures and from the infection mixtures of macrophages and bacteria was performed with the Qiagen RNeasy Mini Protocol. The samples were treated with DNase I solution (RNase-Free DNase Set, Qiagen). Residual DNA contamination of the RNA extracts was estimated using 10 μ l of total RNA samples as templates for 16S rRNA gene real Time PCR without previous reverse transcription. The quantity and purity of the total RNA were measured with a BioPhotometer Eppendorf. Complementary DNA (cDNA) was

generated from total RNA with M-MLV RT according to the manufacturer's instructions (Promega) using 2,5 μ M of each reverse primer and 100 ng total RNA (40 μ l final volume).

Primer and Probe Design

The specific primers and probes for *Brucella ovis dnaK* (accession no. M95799.1), *groEL* (accession no. M82975.1) and 16S rRNA gene (accession no. L26168) were designed in this study with Primer3 software v.0.4.0: dnaK-FP

5'gatgccgacatcgaaaagat3'; dnaK-RP 5'tcttcaccttcaagcgaggt3';

dnaK-P 5'FAM-aatatggcgacaagggttctg-TAMRA3'; groEL-FP

5'ccaaggaagtgcgaactggaa3'; groEL-RP 5'agctcagccacaacttcggt3';

groEL-P 5'FAM-ggcttccaagaccaacgata-TAMRA3'; 16SrRNA-FP

5'cagctcgtgtcgtgagatgt3'; 16SrRNA-RP 5'cagagtgcaatccgaactga3'
and
16SrRNA-P 5'FAM-cgtgctacaatggtggtgac-TAMRA3'.

Controls for Semi-Quantitative and Quantitative RT-PCR

B. ovis 202 grown in TSB pH 7.0 was used as positive control. No-
template controls were used for *in vitro* experiments and RNA of
uninfected macrophage was isolated as a negative control. 16S
rRNA gene (a housekeeping gene) was chosen as internal control
for semi-quantitative RT-PCR analysis for normalization of cDNA
samples. The dilutions of cDNA from 10^0 to 10^{-3} were analysed
with primers for 16S rRNA gene and the cDNA samples were
normalized according to quantified of 16S rRNA amplicons. For
real time quantitative RT-PCR, the 16S rRNA gene, the expression

of which is relatively constant in bacteria, was used as a reference.

Semi-Quantitative PCR

Selected genes were amplified from normalized cDNA samples with specific primers (0.6 μM of each primer). The PCR products were analysed on 1.5% agarose gel and visualized by ethidium bromide staining.

Real-Time Quantitative PCR (qPCR)

Samples were amplified in a 20 μl reaction mixture containing 0.5 μM of each oligonucleotide primer, 0.5 μM fluorescent labelled probe for each gene (*dnaK*, *groEL* and 16S rRNA) and 5

μ l normalized cDNA. Amplification program (iQ5 Real Time PCR System-BioRad): 3 min at 95°C, then 40 cycles of 30 s at 95°, 30 s at 56°C and 30 s at 72°C. The final extension was performed for 3 min at 72°C. Relative transcriptional level was determined by the method of $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001).

Statistics

Three replicates were used for all experiments. The relative transcription of the genes and the quantification of the proteins were calculated as the mean \pm the standard deviation (S.D.)

Macrophage Infection Assay

Macrophage cell infection was assayed as described earlier (Buchmeier and Heffron, 1990). *B. ovis* 202 cells grown on tryptose agar plates for 89 h were harvested by centrifugation at 13.000 rpm. Then, bacteria were opsonized with polyclonal rabbit anti-*Brucella ovis* antibodies for 30 min at 37°C and washed once in PBS. The plates were infected with *Brucella ovis* at a density of 50 bacteria per macrophage and incubated at 37°C in 5% CO₂ and a humidified atmosphere for 60 min. The cells were then washed three times with PBS to remove extracellular bacteria. The cells were incubated for 1 h in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) (Life Technologies, Cat. No. 11320-074) supplemented with 100 µg/ml ampicillin plus 50 µg/ml kanamycin to kill the extracellular still

present bacteria. To ensure that macrophage protein synthesis was inhibited, 50 µg/ml cycloheximide was added to uninfected macrophages. At various time points post infection, the macrophages were lysed with 0,2% (vol/vol) ice-cold Triton X-100 for 30 min and bacterial cells were collected by centrifugation at 13.000 rpm for 15 min. All the infection experiments were performed in triplicate. Two control wells (positive and negative) were stained by conventional Giemsa method.

Results and Discussion

Survival and replication of *Brucella* in host phagocytes are the key components of their virulence (Enright, 1990; Smith and Ficht, 1990). In order to understand any possible contribution of

the molecular chaperones DnaK and GroEL for *Brucella ovis* stress survival and intracellular persistence, the expression in different culture media under different stress conditions were analysed at the transcriptional and translational levels and compared with that of bacteria grown inside murine J774A.1 cells. A proteomic approach was performed by other authors with *B. suis*, *B. abortus* and *B. melitensis* (Lin and Ficht, 1995; Kohler et al., 1996; Teixeira-Gomes et al., 2000) and the proteins implicated in intracellular survival were identified. In the proteomic analysis of *Brucella melitensis*, Teixeira Gomes et al. (2000) found 62 protein spots that showed either increased (DnaK, GroEL, AapJ, RRF, Fe Mn SOD, Cu Zn SOD) or decreased (Alpha-ETF, ClpP, bacterioferritin, BvrR, IalB, and pyruvate dehydrogenase E1 component beta subunit) levels depending of the stress.

The transcription level of the *dnaK* and *groEL* were determined by the combination of semi-quantitative RT-PCR and quantitative real time RT-PCR enabled the sensitive quantification of the gene expression. The selected genes were amplified from normalized cDNA samples (Figure 1).

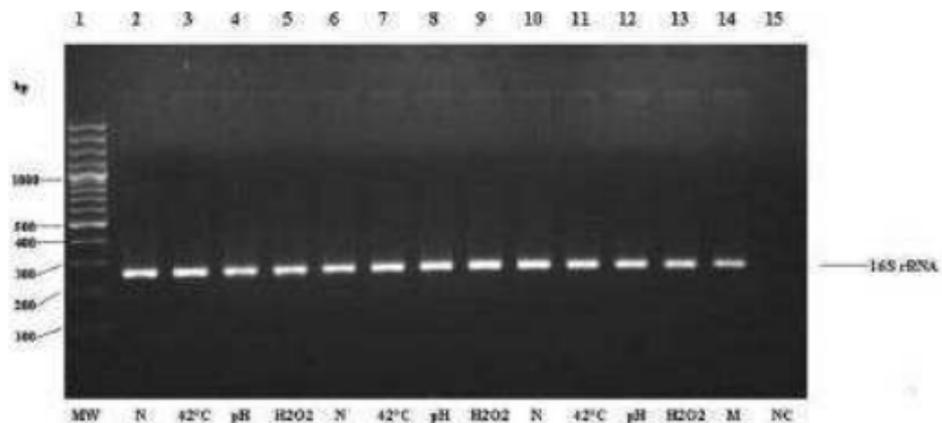


Figure 1: Normalization of the cDNA *B. ovis* Samples according to Quantity of 16S rRNA Products. Lane 1: 100-3000 bp DNA Ladder (MW); Lanes 2-5 (TSB): Lane 2: Normal Conditions (N), Lane 3: Heat Shock (42°C), Lane 4: pH 5.5 (pH), Lane 5: 50 mM H₂O₂ (H₂O₂); Lanes 6-9 (BHI with 5% Glc): Lane 6: Normal Culture (N), Lane 7: Heat Shock (42°C), Lane 8: pH 5.5 (pH), Lane 9: 50 mM H₂O₂ (H₂O₂); Lanes 10-13 (Columbia Agar): Lane 10: Normal Conditions (N), Lane 11: Heat Shock (42°C), Lane 12: pH 5.5 (pH), Lane 13: 50 mM H₂O₂ (H₂O₂); Lane 14: *B. ovis* Infected Macrophages (M); Lane 15: no-Template Control (NC).

Three replicate were used for each *dnaK* and *groEL* real time qRT-PCR experiment and the relative transcription (R) was calculated. The transcription of these two genes was modified under different stress conditions depending on media content (Table 1); the results of Western blot analysis under all the stress conditions used *in vitro* are a confirmation of this. Real Time RT-PCR showed that DnaK and GroEL were induced during macrophage cell infection (Table 1).

Table 1: Transcription of *dnaK* and *groEL* Measured by Real Time qRT-PCR in *B. ovis* in Different Stress Conditions: PC (Positive Control): TSB, Normal Conditions; R: Relative Transcription Determinated by the Method of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) \pm Standard Deviation (S.D.) of Three Replicates.

Sample	Relative transcription (R)					
	<i>dnaK</i> gene			<i>groEL</i> gene		
	TSB	BHI with 5% glucose	Columbia agar	TSB	BHI with 5% glucose	Columbia agar
PC	1 \pm 0.43	1 \pm 1.08	1 \pm 0.31	1 \pm 0.12	1 \pm 0.33	1 \pm 0.33
normal conditions (N)		5.31 \pm 0.9	1.16 \pm 0.41		9.25 \pm 0.44	7.78 \pm 1.06
heat (42°C)	18.5 \pm 0.06	49.18 \pm 0.63	1.3 \pm 0.97	14.92 \pm 0.09	22.78 \pm 0.3	35.26 \pm 0.16
H ₂ O ₂	0.76 \pm 0.65	21.7 \pm 0.07	1.12 \pm 0.46	1.27 \pm 0.32	22.47 \pm 0.33	18.76 \pm 0.41
low pH	18.24 \pm 0.10	340 \pm 0.81	1.40 \pm 0.96	17.87 \pm 0.04	103.96 \pm 0.23	37.27 \pm 0.68
Macrophages	27.28 \pm 0.59			29.24 \pm 0.66		

The results of the real time quantitative PCR of *dnaK* and *groEL* genes were consistent with the observation that the induction of DnaK and GroEL depends on the transcription (Cardoso et al., 2010). The semi-quantitative PCR of the genes has been a less sensitive method that showed the different levels of the transcription, being a partial confirmation of the other two methods results (Figure 2).

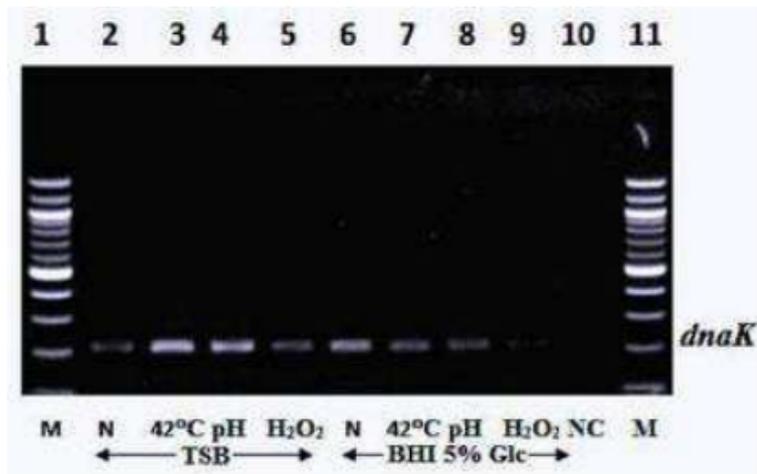


Figure 2: Relative Transcription of *dnaK* Gene in *B. ovis* Grown in TSB and BHI with 5% Glucose by semi-Quantitative RT-PCR. Lane 1 and 11: DNA Molecular Weight Standard Marker (M); Lanes 2-5 (TSB): Lane 2: Normal Conditions (N), Lane 3: Heat Shock (42°C), Lane 4: pH 5.5 (pH), Lane 5: 50 mM H₂O₂ (H₂O₂); Lanes 6-9 (BHI with 5% Glc): Lane 6: Normal Culture (N), Lane 7: Heat Shock (42°C), Lane 8: pH 5.5 (pH), Lane 9: 50 mM H₂O₂ (H₂O₂); Lane 10: Negative Control (NC).

The quantification of the bands intensity for DnaK and GroEL proteins (Figure 3) and the determination of the apparent molecular weight from Western blots were done with UN-SCAN-IT gel™ software.

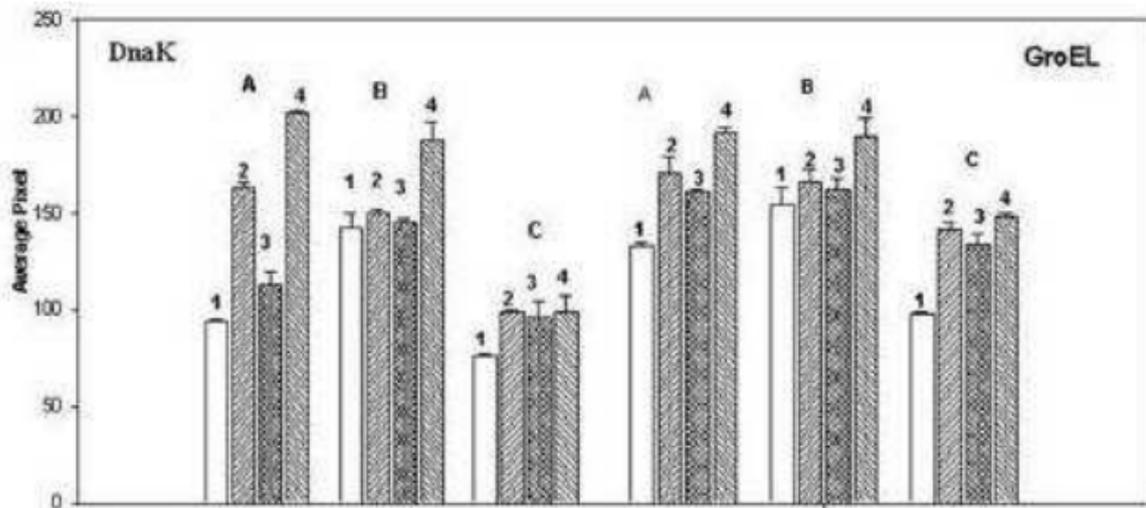


Figure 3: DnaK and GroEL Proteins Quantification by Densitometric Scanning of Western Blot (Average Pixel). A: TSB; B: BHI with 5% Glucose; C: Columbia Agar. The Stress Conditions Induced in Media: (1) None, (2) Heat Shock (42°C), (3) 50 mM H₂O₂ and (4) pH 5.5, Respectively. The Data Presented are Calculated as the Mean \pm the Standard Deviation (S.D.).

The DnaK and GroEL proteins were identified by the Western blot analysis where the proteins with the same molecular mass as DnaK and GroEL cross-reacted with the antibodies specific for these proteins with the strong reactions. These protein spots were accepted as the DnaK (Figure 4) and GroEL (Figure 5).

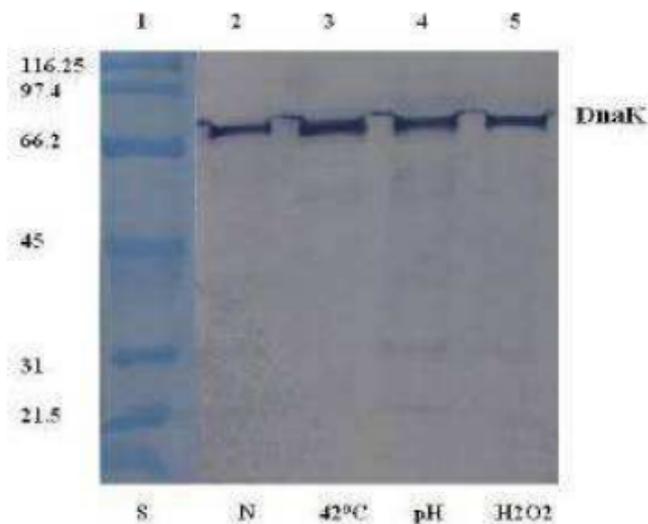


Figure 4: Western Blot Analysis of DnaK Expression in *B. ovis* Cells Grown in TSB. Lane 1: SDS-PAGE Molecular Weight Standard (kDa) (S); Lane 2: Normal Conditions (N); Lane 3: Heat Shock (42°C); Lane 4: pH 5.5 (pH); Lane 5: 50 mM H₂O₂ (H₂O₂).

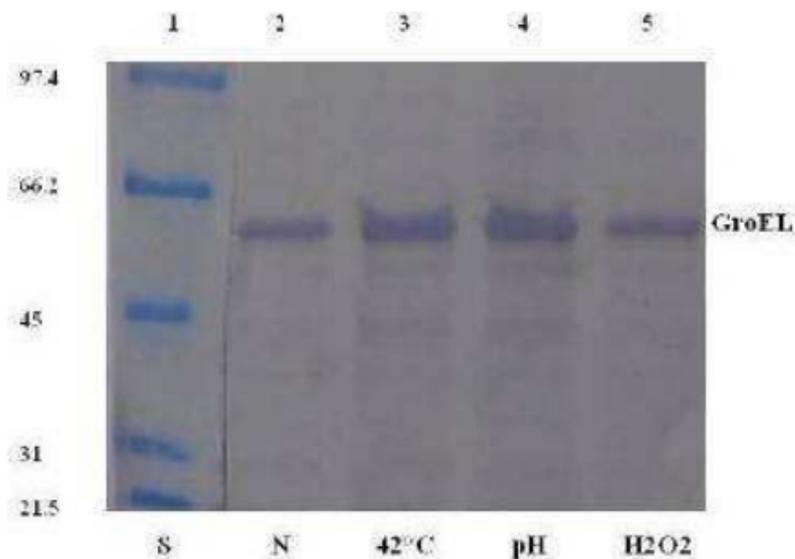


Figure 5: Western Blot Analysis of GroEL Expression in *B. ovis* Cells Grown in TSB. Lane 1: SDS-PAGE Molecular Weight Standard (kDa) (S); Lane 2: Normal Conditions (N); Lane 3: Heat Shock (42°C); Lane 4: pH 5.5 (pH); Lane 5: 50 mM H₂O₂ (H₂O₂).

The results showed an increase of protein level corresponding to DnaK in TSB medium, as a response of *B. ovis* to the heat (Figure 3A). In addition, the DnaK level was increased in BHI medium with 5% glucose after exposure to the increased temperature (Figure 3B). In contrast, the DnaK expression of *B. ovis* on solid medium with peptone (Columbia agar) was unchanged (Figure 3C). The increased induction of *groEL* gene by heat shock was observed regardless of the media composition. The effect of the reduced pH on the chaperone synthesis was estimated by the level of DnaK and GroEL proteins extracted from *B. ovis* cells grown in the TSB, BHI with 5% glucose and Columbia agar, respectively, at pH 5.5 compared with the same media at pH 7.0 (Figure 3). A major increase in the level of these two molecular chaperones was observed at acid-stressed organisms cultured in TSB (Figure 3A) and BHI with 5% glucose (Figure 3B). The

presence of peptone in the solid medium in the absence of glucose (Columbia agar) caused no change in the DnaK expression as comparing with normal pH (7.0). Under the same conditions, the level of GroEL expression was increased (Figure 3C).

SDS-PAGE profiles and Western blot analysis of proteins synthesized in *B. ovis* incubated in TSB medium (Figure 3A) and Columbia agar (Figure 3C) under oxidative conditions show no change in the synthesis of DnaK protein. The high concentration of glucose in medium induced a decrease in DnaK protein synthesis in *B. ovis* exposed to 50 mM H₂O₂ (Figure 3B). The GroEL expression under oxidative conditions was influenced by the media composition. The high concentration of glucose in medium induced an increase in the GroEL protein synthesis

under oxidative conditions (Figure 3B) and a small increase of this molecular chaperone level was noticed in the absence of glucose in the solid medium with peptone (Figure 3C). In contrast, the level of GroEL protein was decreased in TSB medium with 50 mM H₂O₂ (Figure 3A).

GroEL protein is up-regulated when *B. ovis* cultures were grown on media with peptone regardless of glucose under all stress conditions. It seems that too much peptone (relative to glucose) present in the medium increases the pH by the overproduction of ammonium stimulating only the GroEL expression. Under the similar alkaline conditions, the DnaK expression was unchanged, suggesting the response may be shifted by the medium.

Our results showed a poor correlation between the relative levels of transcription for *dnaK* (Figure 2) with real time quantitative PCR values (Table 1) in *B. ovis* cells grown in TSB and BHI media under various stress conditions. It seems that *dnaK* transcript level (and also *groEL*) is higher in the BHI broth with 5% glucose as compared to TSB. This certainly indicates a significant effect of this BHI medium in the induction of the expression of chaperone genes under acidic pH and heat shock in *B. ovis*, but less against oxidative conditions. This could reflect the increased osmolarity of this medium due to extra glucose supplementation, a stressor promoting this effect in other bacteria, as mentioned above.

B. ovis is a fastidious organism with a low growth and metabolic activity (Tsolis et al., 2009; Al Dahouk et al., 2010). Meyer (1969) showed that *B. ovis* is defective in oxidative metabolism of

glucose and other sugars, this finding being confirmed by the recent studies that show the inability of *B. ovis* to use glucose as a primary carbon source, suggesting a correlation between this metabolic deficiency and the narrowing of the tissue tropism and host range of *B. ovis* (Tsolis et al., 2009; Al Dahouk et al 2010). However, McAlpin and Slanetz (1928) showed that all species of *Brucella* except *B. abortus* are able partly to use the available glucose in the medium, the degree of acid production varied with the different strains (Zobell and Meyer, 1930). In this context, the high levels of *dnaK* and *groEL* transcripts in BHI medium with 5% glucose could be explain by the acidification of the medium, probably by the use of glucose in the fermentation process. As a consequence, these two proteins in *B. ovis* might be involved in the adaptive acid tolerance response.

In this study, using various media for *B. ovis* culture under stress conditions we have shown that the *B. ovis* stress response was significantly influenced by the media composition. *dnaK* and *groEL* transcript levels are higher under reduced pH and heat shock in rich medium (BHI broth with 5% glucose) than in minimal medium (TSB); similar data were reported by Lin et al. (1995b) and Foster et al. (1991) regarding the response other pathogens to the acidic stress.

The comparison of the DnaK and GroEL expression in *B. ovis* cultures exposed to various environmental stress conditions allowed us to distinguish different levels of these two molecular chaperones expression. The present study revealed that environmental stress rapidly modified the DnaK and GroEL expression in *B. ovis*. The relative transcription level of the *dnaK*

and *groEL* genes change under in vitro stress conditions examined, also during macrophage infection. Our results demonstrate that heat, the acid pH and intramacrophagic cell infection induce the expression of DnaK and GroEL in *B. ovis*, confirming the previously described data on other *Brucella* species involved in human pathology, as mentioned above. The pH reductions are mainly responsible for the induction of these genes inside phagocytic cells providing that the temperature is not changes. *dnaK* and *groEL* genes may play possibly an important role in the resistance against the bactericidal activity of macrophages and against the pathogenicity of *B. ovis*.

The expression of molecular chaperones genes in bacteria is controlled at transcriptional level by both positive and negative mechanisms. In *Escherichia coli*, the induction of the majority of

up-regulated cytoplasmic heat shock proteins occurs through the sigma factor (σ^{32}) located upstream of heat-shock genes (Chuang et al., 1993). The identification of putative heat-shock promoter -35 and -10 consensus sequence for *B. melitensis*, *B. abortus* and *B. ovis* suggest that σ^{32} could be responsible for the increased cellular levels of DnaK and GroEL expression in *Brucella* (Cellier et al., 1992; Lin et al., 1992; Teixeira-Gomes et al., 2000). Delory et al. (2006) identified a RpoH-like sigma factor of the RNA polymerase (σ^{H2}) with multiple roles in the adaptation of *B. melitensis* to heat, cold and oxidative stress by the control of *dnaKJ* and *groESL* operons. These findings could be the potential explanations for the increased cellular levels of DnaK and GroEL in *B. ovis* inside macrophages and under various stress conditions.

In addition, the negative heat shock regulation appears to be more prominent in bacteria than positive regulation by σ factors. The identification of any putative CIRCE element upstream of the *groES* operon, but not upstream the *dnaKJ* operon (Narberhaus, 1999) could explain the differential regulation observed between these two operons in *B. ovis*.

This study suggests that the molecular chaperones, DnaK and GroEL, are regulated at the transcriptional and translational levels by various stress conditions and that this regulation may help improving the resistance of *B. ovis* cells to environmental stress, including the macrophage microbicidal mechanisms. Our data represent the first transcription analysis of the molecular chaperones in a non-zoonotic *Brucella* species, regarding the

resistance of *B. ovis* cells in macrophages and to various environmental conditions.

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