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Heat shock stress: Profile of differential expression in *Corynebacterium pseudotuberculosis* biovar Equi

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ABSTRACT

Transcriptome studies on *Corynebacterium pseudotuberculosis* have recently contributed to the understanding about this microorganism's survival mechanisms in various hostile conditions. The gene expression profile of the *C. pseudotuberculosis* strain 1002 (Ovis biovar), has revealed genes that are possible candidates responsible for its maintenance in adverse environments, such as those found in the host. In another strain of this bacterium, 258 (Equi biovar), a high temperature condition was simulated, in order to verify which genes are responsible for promoting the persistence of the bacterium in these conditions, since it tolerates temperatures higher than 40 °C, despite being a mesophilic bacterium. It was possible to generate a list of genes using RNAseq technology that possibly contribute to the survival of the bacteria in this hostile environment. A total of 562 genes were considered as differentially expressed, then, after the fold-change cutoff, 113 were considered induced and 114 repressed, resulting in a total of 227 genes. Therefore, hypothetical proteins presented a fold change above 6, and genes characteristically in control for this type of stress, such as *hspR*, *grpE*, and *dnaK*, presented a fold change above 3. The *clpB* gene, a chaperone, drew attention due to presenting a fold change above 3 and located in a pathogenicity island. These genes may contribute towards efficient solutions to the effects caused by ulcerative lymphangitis in equines, thus attenuating the damage it causes to agribusiness.

1. Introduction

Bacteria are subject to several types of environmental stresses, such as changes in temperature, nutrient availability, pH variation, and presence of toxic molecules. Consequently, the mechanisms used by the bacterium to respond and protect itself from different types of stresses are essential for its survival and proliferation. In most cases, bacterial responses lead to the activation of genes, whose products act as a response to a given physical-chemical stress. Factors regulating these genes answer to specific signals, environmental or cellular, by stimulating or inhibiting transcription, translation, or any other event of gene expression, in order to appropriately modify the rate of synthesis of the gene products, obtaining a physiological and biochemical adaptation (Dow and Daniels, 2000). This way, they can adhere, invade, replicate and escape from the immune system, causing the host diseases.

It is extremely important to detect the genes responsible for the microorganism maintenance in precarious situations to prevent the onset of the disease triggering process. A disease that has been causing great damage, especially to echinoculture, is the ulcerative lymphangitis. This globally distributed disease is considered to be moderately contagious and it has low mortality rate, however, it is characterized by lower limb lymphatic vessels lethargy, anorexia, ulcers in various parts of the body and weight loss (Abu-Samra et al., 1980). Treatment with antibiotics, aside from being expensive, is not the most recommended, because they do not efficiently penetrate the rigid capsule of the abscess, and because the bacterium is located intracellularly during infection (Collet et al., 1994).

The most common infectious agent known to cause this disease is the microorganism *Corynebacterium pseudotuberculosis*; a Gram positive, facultatively intracellular bacterium, belonging to the phylum

Abbreviations: Cp258, *Corynebacterium pseudotuberculosis* 258; RT, room temperature; BHI, Brain Heart Infusion; rpm, rotation per minute; FPKM, fragments per Kilobase of Exon per Million Fragments Mapped

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Actinobacteria, that causes different chronic diseases which affects a variety of hosts, including humans, thus causing mainly great damage to agribusiness (Dorella et al., 2006). Two biotypes of *C. pseudotuberculosis* are recognized based on the synthesis of nitrate reductase (Batey, 1986). The ability to reduce nitrate to nitrite (nitrate positive) is a characteristic of strains that preferentially infect equines, cattle and buffaloes (biovar Equi), while the strains that preferentially infect goats and sheep (biovar Ovis) are nitrate negative.

In order to achieve better biological understanding of this bacterium, a large number of genomes are being deposited in the NCBI database, which has allowed genomic and postgenomic studies essential to understanding the organism (Folador et al., 2016; Pinto et al., 2014; Santana-Jorge et al., 2016; Silva et al., 2017a; Soares et al., 2013), providing precise results that may contribute to reaching an effective solution against the effects of the different diseases caused by this microorganism.(Silva et al., 2017b), in the field of proteomics, detected proteins that infer a difference between biovars, in relation to virulence, and other proteins, that have been studied for a longer time, such as phospholipase D, are found in greater abundance in biovar Equi, in relation to Ovis. The obtained proteins can contribute to a better physiology and virulence understanding of each biovar.

It is known that the species presents several virulence factors that favor its persistence in unfavorable conditions, however, these factors have not been used successfully to develop vaccines, diagnosis techniques, or therapy to date. Among the postgenomic studies carried out, we highlight the transcriptome study of the biovar Ovis strain, *C. pseudotuberculosis* 1002, the first deposited genome of the species (Ruiz et al., 2011), that demonstrated resistance to various abiotic stresses, such as acidity, high temperature, and osmotic stress. The authors pointed different genes that may contribute to the persistence of the bacteria. In thermal stress, the *hspR* gene, encoding heat shock regulatory protein, showed a fold change above 4, standing out as one of the main candidates supporting the bacteria in its host environment (Pinto et al., 2014).

Thermal shock is a form of stress that the bacterium commonly faces when the host, in the process of infection, displays a protective response, thus raising the temperature (Hasday et al., 2014). The bacterium then, attempting to survive, alters its own genetic expression, directing it to a rapid and adaptive response to the environment. The growth of *C. pseudotuberculosis* can be observed in temperatures ranging from 14 °C to 40 °C, but its optimum growth occurs at a temperature of 37 °C (Dorella et al., 2006) and the body temperature of horses, for example, can vary from 37,2 °C to 38,2 °C. In the process of infection, the host's temperature rises, jeopardizing the bacterium's replication and survival in the medium. The bacterium, however, is able to escape through its virulence factors and gene expression control, and it can impose itself upon the host. In many pathogens, virulence determinants are under control of transcriptional activators that respond to increasing temperature fluctuations, leading to the expression of virulence genes at 37 °C, as in L. *monocytogens*.

In L. *monocytogens*, a Gram-positive intracellular pathogen, the activation of virulence genes is controlled by the transcriptional activator *prfA* (de las Heras et al., 2011). At 30 °C, *prfA* is expressed as monocistronic transcript. Moreover, at 37 °C, in addition to the monocistronic transcript, the *prfA* gene is also transcribed by a different promoter as a bicistronic message including *prf* and *pic* genes encoding proteins (phospholipase C phosphatidylinositol-specific) that act to disrupt the host's membrane by lipid hydrolysis. Therefore, the *prfA* gene is transcribed by different promoters depending on different temperatures (de las Heras et al., 2011).

The strain 258 was incubated at a temperature of 50 °C *in vitro* to evaluate the influence of thermal shock on bacterial replication, also to verify the transcription profile of the organism and visualize the direction taken by the microorganism to survive in a hostile environment. It is necessary to search for new factors and improve our current biological understanding of the organism, to minimize or eradicate the effects of the related diseases. Hence, we propose the identification of genes, induced by the stress condition, using RNAseq technology.

2. Materials and methods

2.1. Bacterial cultures and application of stress conditions

The strain 258 of *Corynebacterium pseudotuberculosis*, biovar Equi (isolated from horse in Belgium) was isolated on Brain Heart Infusion (BHI) plates, containing 200 g calf brain infusion, 250 g beef and heart infusion, 10 g peptone protease, 2 g dextrose, 5 g sodium chloride, and 2,5 g disodium phosphate; pH 7.4 ± 0.2 at 25 °C, room temperature (RT). A colony was inoculated in 20 ml of BHI broth supplemented with Tween® 80 (0.05%) in a Falcon tube for 24 h at 37 °C in a shaker under 140 rpm. Afterwards, the sample was submitted to a 1/100 parts dilution, by preparing a fresh inoculum in sterile BHI broth plus Tween® 80 and incubated once again under 140 rpm, being monitored until the exponential phase onset (A_{600} = 0.2). After reaching this stage, the inoculum was divided in two cultures, one remaining at 37 °C and the other placed in a 140 rpm shaker at 50 °C for 15 min.

An aliquot was serially diluted from 10^{-1} to 10^{-6} and the dilutions 10^{-4}, 10^{-5}, and 10^{-6} were plated onto BHI agar plates and incubated at 37 °C for 48 h, to verify cell viability by counting colonies. To analyze the statistical differences of cell viability, a student's *t*-test was applied. Statistical significance indicates a *p*-value inferior to 0.05. The rest of the culture was centrifuged at room temperature at 8000 rpm for 3 min, and the resulting pellet was resuspended in 2 ml of RNAlater® (Ambion, USA), as recommended by the manufacturer.

2.2. RNA extraction

The total RNA was extracted using the ChargeSwitch® Total RNA Cell kit (Invitrogen), according to the manufacturer's recommendations, albeit with some modifications. In the cell lysis step, glass microbeads 1 mm in diameter (Bertin Technologies) were added into the 2 ml tubes containing the samples, and the tubes were homogenized through the use of the Precellys 24 homogenizer, for two 15-s cycles at 6500 rpm, with a 30-s interval between cycles. The samples were, then, centrifuged for one minute, with the supernatant being transferred to new 2 ml tubes and incubated in a dry bath at 60 °C for 15 min. DNAse was added to eliminate any genomic DNA contamination, and total RNA was eluted in 100 µl of RNAse-free milli-Q water, and quantified using a Qubit® 2.0 fluorometer (Invitrogen, USA).

2.3. rRNA depletion for mRNA enrichment

The ribosomal RNA depletion was performed *via* the Ribominus™ Transcriptome Isolation kit for yeast and bacteria (Invitrogen, USA), according to the manufacturer's recommendation to enrich the mRNA sample.

2.4. SOLiD™ V3 sequencing

To build the cDNA library from rRNA-depleted total RNA, cDNA synthesis was performed using the SOLiD™ V3 Total RNA-Seq kit (Applied Biosystems, USA) according to the manufacturer's instructions, and the material prepared was quantified using a Qubit® 2.0 fluorometer (Invitrogen). The RNA was fragmented using RNase III. Then, the fragments were hybridized using barcodes at both ends and amplified by reverse transcription according to the protocol provided by the SOLiD™ Total RNA-Seq kit (Life Technologies™, CA). The fragments ranging in size from 150 to 250 base pairs were selected using 6% polyacrylamide gel electrophoresis of cDNA amplified by PCR. Subsequently, the cDNA was purified using the PureLink™ PCR Micro kit (Invitrogen, USA) and quantified using a Qubit® 2.0 fluorometer (Invitrogen, USA). The samples were confirmed by 2% agarose gel