Original Article

Molecular analysis of the adaptive response in *Salmonella* Typhimurium after starvation in salty conditions

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Abstract

Introduction: The pathogenic bacterium *Salmonella enterica* serovar Typhimurium elicits a variety of genetic programs to adapt to stress conditions encountered within hostile environments such as host phagocytes and preserved food.

Methodology: In this work, differential display (DD) methodology was used to investigate the effect of one month starvation in a salty microcosm (0.5 M NaCl) on transcript profiling in a *Salmonella* Typhimurium LT2 strain. cDNA fragments resulting from differentially expressed mRNA were eluted from the gel, re-amplified, cloned, and then sequenced.

Results: A total of 21 differentially expressed bands were detected by DD reverse transcription-polymerase chain reaction (RT-PCR). However, only 12 of them were successfully identified as upregulated genes in stressed cells. Based on the sequencing data and BLAST analysis, these genes were *sopA*, *ssaD*, *yhhK*, *gmK*, *cspC*, *uspA*, *ompR*, *phoP*, *stcC*, *fimA*, *acrA*, and *yehZ*. As a confirmation of the differential expression, RT-PCR was carried out using a set of specific primers. Remarkably, the expression levels of these genes were significantly increased in starved bacteria compared to standard laboratory conditions.

Conclusions: Our results indicate that the starvation of *Salmonella* Typhimurium over one month in a salty microcosm changes the expression of stress proteins, response regulator in a two-component system, outer membrane proteins, effector proteins translocated by *Salmonella* pathogenicity island SPI1 and SPI2 type III secretion systems (TTSS), several metabolic enzymes, efflux pumps, and transport proteins. This suggests that the expression of the identified genes is important for the response of this pathogen to starvation in salt.

Key words: *Salmonella*; starvation; salt stress; gene expression; DDRT-PCR.

Introduction

The genus *Salmonella* is one of the most significant enteric pathogens, causing serious human diseases such as gastroenteritis, septicemia, osteomyelitis, pneumonia, meningitis, and arthritis [1,2]. Particularly, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a common food-borne pathogen and frequent cause of gastroenteritis in humans [3]. Although non-typhoid *Salmonella* infections do not tend to be life threatening for healthy individuals, *S.* Typhimurium can cause deaths in elderly, very young, and immunocompromised patients [4].

Traditionally, salting had been considered an effective way to reduce microbial spoilage of foods by producing a bacteriostatic or bactericidal condition [5]. Indeed, salt treatment is part of the hurdle system to control pathogens. Frequently, osmotic conditions are used for food preservation and to control hazardous bacteria such as *Salmonella* [6]. However, *S.* Typhimurium can be isolated from shellfish [7], and can even persist and survive for long periods in hyperosmotic environments such as seawater [8].

S. Typhimurium responds to various stimuli such as oxidative stress, extreme pH, heat shock, osmotic conditions, and starvation by changing the expression of groups of genes termed "stimulons" [9]. Genes that contribute to the fitness of the pathogen within the host have been classified as virulence factors [10,11]. Several virulence factors are involved in the adaptive response to environmental conditions because pathogens are exposed to a variety of stresses [10]. Studies using mice, together with *in vitro* models such as cultured eukaryotic cells, have yielded details about

the molecular and genetic events that underpin some aspects of *Salmonella* virulence [12,13]. Type III secretion systems (TTSSs) are used by *Salmonella* to secrete and translocate a variety of effector proteins from the bacteria directly into the host cell cytosol. Inside the host cell, these proteins elicit a range of effects that aid bacterial invasion and survival. Many *Salmonella* TTSS effector proteins have been characterized and contribute to invasion of epithelial cells and enteropathogenic responses in the early stages of *Salmonella* infection [14].

With the onset of molecular biology techniques, several strategies such as differential hybridization [15], microarray [16], *in vivo* expression technology [17], signature-tagged mutagenesis [18], and differential display reverse transcription-polymerase chain reaction (DDRT-PCR) [19] have been used to identify stress-associated genes in bacteria. The DDRT-PCR method was originally described by Liang and Pardee [20] as a fingerprinting technique to identify and compare mRNA expressed during different physiological conditions. It involves the amplification of DNA fragments from the mRNA by reverse transcription (RT) using olig-T or hexamers primers followed by PCR amplification using arbitrary primers [20,21]. The method can be used as random arbitrary primed-PCR (RAP-PCR), where arbitrary primers initiate the RT of the mRNA at random sites [22]. DDRT-PCR is reported to be highly reproducible and very sensitive [20].

In this study, we investigated the combined effects of one month of starvation and high salt concentration (0.5 M NaCl) on gene expression of *S*. Typhimurium LT2 strain using the DDRT-PCR method.

Methodology
Bacterial strains and culture conditions
Bacteria were routinely subcultured on tryptic soy agar (TSA, AccumixTM, Verna-Goa, India, Cat. No. AM5091) plates from frozen stocks. The overnight cultures in Luria-Bertani broth (Accumix TM, AM50572) at 37°C and 100 rpm were used to prepare inoculation. For stress conditions, *S*. Typhimurium LT2 cells were grown for 24 hours at 37°C in 100 mL LB broth prepared with 0.5 M NaCl in closed 250 mL laboratory bottles. The cultures were then stored for one month (from 4 March 2013 to 2 April 2013) in a laboratory cabinet at room temperature(generally between 20°C and 25°C) in obscurity, without being shaken. The experiment was performed in three independent replicas.

Differential display RT-PCR
Total RNA was isolated using the SVTotal RNA Isolation System (Promega, Madison, WI, USA), following the manufacturer's instructions, from fresh culture of the reference strain *S*. Typhimurium LT2 (overnight culture in LB broth at 37°C). To investigate the effect of one month starvation in a salty microcosm on transcriptional profile, total RNA was extracted directly from the old culture containing the starved *S*. Typhimurium cells. To remove any eventual genomic DNA contamination, RNA samples were purified using a Message Clean Kit (GenHunter, Nashville, USA). For the reverse transcription reaction, 1 μgof total RNA, 1 μL of random hexamers (3 μg/μL), and 100 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) were used. The RT reaction was carried out for 60 minutes at 40°C in a MultiGene gradient thermal cycler (Labnet International, Woodbridge, USA). To avoid detecting DNA contamination in DDRT-PCR products, one control was used for each RNA sample without MMLV reverse transcriptase. Arbitrary primers set of 13-Mer (GenHunter) was used for cDNA amplification as described previously [19]. The amplification conditions were 5 minutes at 94°C, 40 cycles of 1 minute at 94°C, 2 minutes at 40°C and 30 seconds at 72°C, followed by 5 minutes at 72°C for final elongation. The DDRT-PCR products were electrophoresed in a 2% agarose gel stained with ethidium bromide.

Selection of differentially expressed bands, re-amplification of cDNA, and cloning system
The differentially expressed genes in stressed *S*. Typhimurium LT2 were isolated by cutting out the bands of interest from low melting point agarose gel, and then purifying cDNA using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Amherst, USA) according to the manufacturer's instructions. Re-amplification of the purified cDNA fragments was performed with the same arbitrary primer combinations and PCR conditions. PCR products were cloned into the PCR-TRAP cloning system (GenHunter) using a PCR-TRAP vector and *E. coli* GH strain. The mechanism of this cloning system involved the phage lambda repressor gene *cI*, which has been incorporated into the vector. The product of the *cI* gene turns off the promoter, which drives the tetracycline resistance gene on the plasmid. Therefore, cloning the DDRT-PCR product directly into the *cI* gene leads to the inactivation of the repressor gene and thus the expression of the tetracycline resistance gene.

The screening by colony PCR was carried out using PCR-TRAP vector-specific primers gh-F (5'-CGACAACACCGATAATC-3') and gh-R(5'-GACGCGAACGAAGCAAC-3') flanking the cloning site into the*cI* gene. PCR parameters are as follows: 94°C for 5 minutes followed by 30 cycles of 30 seconds at 94°C, 40 seconds at 52°C, and 1 minute at 72°C. The colony PCR product should be larger than 120 bp due to the flanking *cI* gene fragment containing the unique cloning site. The clones selected for sequencing were re-streaked onto Luria-Bertani agar (Accumix TM, AM50571) plates containing tetracycline for single colony isolation.

Identification of the differentially expressed cDNA fragments by sequencing

Recombinant PCR-TRAP plasmids were extracted from overnight selected clone culture using EZ-10 spin column plasmid DNA Minipreps (Bio Basic, Amherst, NY, USA). A part of the recombinant plasmid was sequenced using gh-F and gh-R primers (120 bp fragment of the *cI* gene flanking the cDNA insert). DNA sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, USA) using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Finally, DNA sequence homology search within the GenBank database was performed using BLAST.

Semi-quantitative RT-PCR

The confirmation of the detected variable gene expression was done by RT-PCR, using specific primers. Based on the sequencing data, the design of primers was carried out using Primer3 version 4.0 available online at http://bioinfo.ut.ee/primer3-0.4.0/. Primers were chosen according to the following parameters: length between 20 and 22 bases (optimal 20 bases), melting temperature (Tm) between 60°C and 65°C (optimal Tm 62°C), and length of amplification product between 120 and 300 bp.

Total RNA was isolated from both fresh culture of the *S.* Typhimurium LT2 (cultivation in LB broth at 37°C) and old culture of the starved *S.* Typhimurium cells. In order to evaluate the differences in starting mRNA template between the two studied conditions, the number of PCR cycles was between 21 and 30, depending on the used specific primer pair. It is critically important to select the appropriate number of cycles, so that the amplification product is clearly visible on an agarose gel and can be quantified, but also so that amplification is in the exponential phase

Table 1. Primers used in semi-quantitative reverse transcription-polymerase chain reaction (*rrsG* was used as housekeeping gene).

Gene	Primers sequence(forward and reverse)	Product size(bp)	Reference
ssaD	5'-GGTTGGCTGGCGTATTCTTG-3' 5'-CTGCAGGCTGCCATCTTCTT-3'	167	This study
sopA	5'-TGGACTGAGAACGCTGTGGA-3' 5'-GTGGGCCAGTACGCTTACCA-3'	207	This study
yhhK	5'- GCTGGATTCCTTACGCGTGC-3' 5'- CACGCTGCGGTCTTCAACAC-3'	136	This study
gmK	5'- TTTCTGGAGCACGCGGAAGT-3' 5'- CTTGCGGCATCTTTTCGCGA -3'	142	This study
cspC	5'- AACAGCAGCCGGACCTTTCT -3' 5'- CGGCAGCAAAGATGTGTTCGT-3'	121	This study
uspA	5'- CGCAGTTGATCTCTCCCCGG -3' 5'- AGCGTTGGTAGACAGCTCGG -3'	208	This study
ompR	5'- CTTCACGGAACATCTCGCGC -3' 5'- CGGCGAAGGGTGAAGAGGTT -3'	219	This study
phoP	5'- GGCGTGAGAGATCCACCTGG -3' 5'- GGGGCCGATGACTACGTGAC -3'	136	This study
stcC	5'- GCCGCTACGTCCCCAGTAAT -3' 5'- TTGGTCTGGCTGCGTATCGG -3'	242	This study
fimA	5'- TTGCGGCTGATCCTACTCCG-3' 5'- GCAGAGGAGACAGCCAGCAA -3'	283	This study
acrA	5'- CGCCTGACCGTTCTGTACCA -3' 5'- AGCAAAAGCCGCCGTTGAAA -3'	124	This study
yehZ	5'- TGATCGTGACCGCCGTATCG -3' 5'- ACGTCAGGACATCGCGGAAA -3'	209	This study
rrsG	5'-GTTACCCGCAGAAGAAGCAC-3' 5'- CACATCCGACTTGACAGACC 3'	123	[19]

and has not reached saturation yet. Therefore, the mRNA concentration can be determined from the kinetics of the cDNA amplification during PCR by titration for the first reaction cycle with detectable fluorescence on the gel [23]. A PCR product becomes visible by ethidium fluorescence during the late exponential phase of PCR.

To do this, the cDNA was first synthesized using MMLV reverse transcriptase (Promega). The RT mixture contained 100ng of total RNA, 1 µL specific reverse primer (20 µM) (Table 1), 4 µL of 5x reaction buffer, and 0.5 µL dNTP (50mM). The final volume was adjusted to 20 µL by adding diethylpyrocarbonate-treated water (DEPC-H2O). The mixture was first heated for 5 minutes at 65°C, and then incubated for 60 minutes at 42°C. Finally, the reaction was stopped by incubation for5 minutes at 70°C.

Then, cDNA was amplified by PCR in a 25 µL reaction volume: 5 µL 5x reaction buffer (Promega), 1 µL MgCl2 (50 mM), 0.5 µL dNTP (50 mM), 1 µL of each forward and reverse specific primer (20 µM) (Table 1), 0.2 µL Taq DNApolymerase (Promega, 5U/µL), and 1µLl reverse transcription product. The reaction was performed in triplicate.

After an initial denaturation step at 94°C for 5 minutes, PCR was run in cycles of 1 minute at 94°C, 40 seconds at 58°C, and 40 seconds at 72°C (exceptionally, the annealing temperature was 59°C for *phoP*). The *rrsG* gene was used as a housekeeping gene for the normalization of the transcription variation [24]. A total of 4 µL of the RT-PCR product was electrophoresed in 1.2% agarose gel stained with ethidium bromide and photographed under ultraviolet transillumination GelDoc INGENIUS (SYNGENE). Quantitative analysis of cDNA bands was performed using imaging software (GeneTools, Syngene). Statistical significance was determined by Student's t-test at $p < 0.05$.

Results

Depending on the 13-mer primer combination used, the number of bands generated by DDRT-PCR was variable. There were no bands present in the negative controls, confirming the absence of genomic DNA contamination in the RNA samples. The results showed that 21 differentially expressed bands were detected by DDRT-PCR in *S.* Typhimurium. Only 12 of them were successfully excised from the gel, re-amplified, cloned into PCR-TRAP vector, and then

Figure 1. Agarose gel electrophoresis of colony-PCR products.
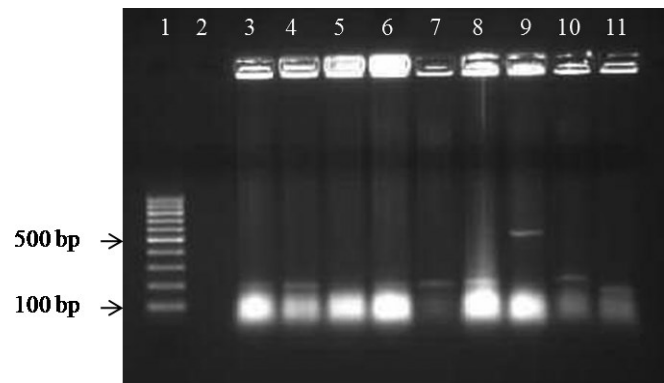
Screening of positive clones by colony PCR using gh-F and gh-R primers located 60 bp away from either side of the cloning site. PCR product larger than 120 bp represents a positive clone. Line 1: 100 bp DNA ladder; 2: negative control; lanes 3 to 11: PCR on colonies.

sequenced. The screening of the positive clones was performed using colony PCR (amplification of 120 bp fragment from PCR-TRAP vector flanking the cDNA insert). APCR product larger than 120bp represented a positive clone (Figure 1). Finally, the recombinant plasmids were extracted from an overnight culture and then sequenced.

After sequencing, 12 cDNA fragments were identified in the stressed *S.* Typhimurium LT2 cells. Based on the sequencing data and BLAST analysis, the detected genes were identical to*sopA*, *ssaD*, *yhhK*, *gmK*, *cspC*, *uspA*, *ompR*, *phoP*, *stcC*, *fimA*, *acrA*, and*yehZ* (Table 2).

Several virulence factors were upregulated in *S.* Typhimurium LT2 after one month of starvation in a salty microcosm. The *sopA* gene coding for TTSS effector protein SopA while *ssaD* coding for TTSS protein SsaD. Two genes coding for stress proteins were also upregulated (UspA, an universal stress protein and CspC, a cold shock protein). The *ompR* gene coding for OmpR (a response regulator in a two-component system with EnvZ) and the *phoP* gene coding for PhoP (a response regulator in a two-component system with PhoQ) were significantly overexpressed.

Moreover, the overexpression of putative acetyl transferase YhhK encoded by the *yhhK* gene and the guanylate kinase GmK encoded by the*gmK* gene are described. Finally, the overexpressed genes *yehZ*, *stcC*, *acrA*, and *fimA* encode, respectively, a putative ABC superfamily transport protein, a putative outer membrane protein, an acridine efflux pump, and a major type 1 subunit fimbrin.

In order to confirm the variable expression, a semi-quantitative RT-PCR was performed. A significant overexpression was detected for all the selected genes in the stressed condition (data shown only for two genes). Figure 2 represents the relative expression of *sopA* and *ssaD* genes coding, respectively, for TTSS effector protein SopA and type III secretion protein SsaD. For normalization, the*rrsG* was used as a housekeeping gene. The results show that the expression level of this reference gene was stable under the two studied conditions (control versus stress).

Discussion

Salmonella enterica serovar Typhimurium is one of the prominent bacterial pathogens that can persist under various or extreme environmental conditions. It responds to high salinity and extended starvation by using a number of adaptive survival mechanisms mediated by several changes in the pattern of gene expression. In this investigation, we attempted to ascertain the effect of starvation over one month in a salty microcosm on the transcriptomic regulation of this pathogen.

The response regulator in the two-component system is one of the major modes of signal transduction that bacteria use to sense and respond to the environment [25]. Obviously, our results demonstrated that *ompR* (a gene coding for OmpR, a response regulator in a two-component system with EnvZ) and *phoP* (a gene coding for PhoP, a response regulator in a two-component system with PhoQ) were significantly overexpressed in the starved *S.*

Figure 2. Verification of the differentially expressed genes. Example of the differential expression confirmation of two identified genes in *S.* Typhimurium LT2 strain by RT-PCR. *rrsG* represents the housekeeping gene.
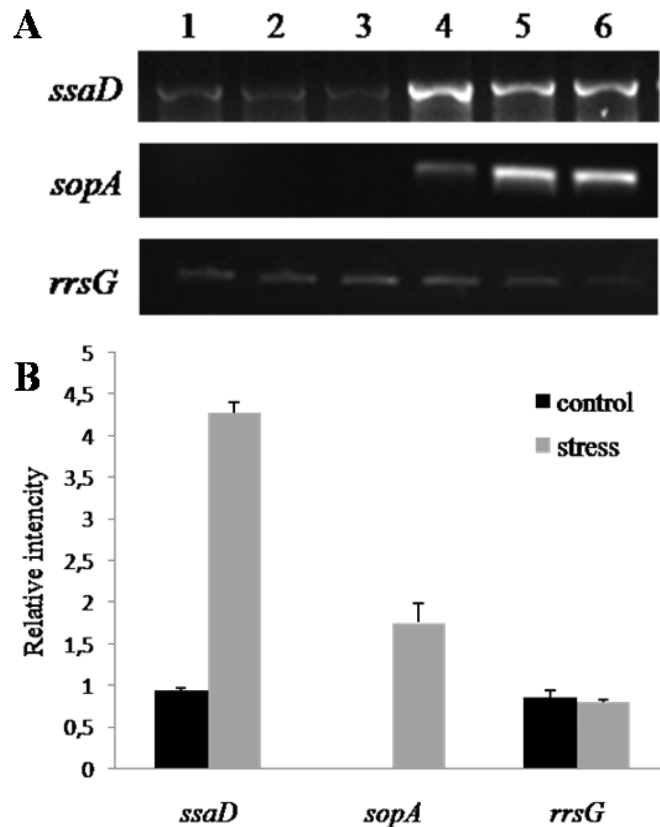
(a) Agarose gel analysis of *ssaD*and*sopA* expression, results shown in triplicate. 1, 2 and 3: strain before starvation; 4, 5 and 6: strain after starvation in a salty microcosm; (b) The relative expression of *ssaD* and *sopA* genes. Columns represent the mean of three values of independents replicas. The whiskers represent the standard error.

Table 2. Genes differentially expressed in starved *S. enterica* serovar Typhimurium LT2 strain.

Identified genes	Function/description
Virulence factors	
sopA	Type III secretion system effector protein SopA
ssaD	Type III secretion system protein SsaD
Enzymes/metabolism	
yhhK	Putative acetyl transferase
gmK	Guanylate kinase
Stress proteins	
cspC	Cold shock protein
uspA	Universal stress protein
Response in two-component regulatory system	
ompR	Response regulator in two-component system with EnvZ
phoP	Response regulator in two-component regulatory system with PhoQ
Outer membrane proteins and transport proteins	
stcC	Putative outer membrane protein
fimA	Major type 1 subunit fimbrin (pilin)
acrA	Acridine efflux pump
yehZ	Putative ABC superfamily transport protein (bind-prot)

Typhimurium cells. The two components that characterize these systems consist of a histidine kinase and a response regulator, which are usually associated with signal detection and output control, respectively [26]. In response to a number of environmental signals, PhoP and OmpR regulate expression of a network of genes involved in virulence and survival of *Salmonella* [27,28]. Thus, our study showed an increase in the expression level of two virulence factors in stressed cells: SopA is a TTSS effector protein and SsaD is a TTSS protein. The *ssaD* gene was also referred to as *spiB* [29]. The identified *Salmonella* virulence genes are located in SPI clusters and may have defensive functions [30]. SopA belongs to the *Salmonella* pathogenicity islands SPI1 TTSS system while SsaD belongs to SPI2. Other works have reported that the expression of SPI1 and SPI2 genes is highly regulated and related to the change of environmental conditions, such as osmolarity and pH [31,32].

Prominent among the adaptive response in *Salmonella* is the expression of stress proteins with chaperone machinery [33]. Herein, we described the overexpression of two stress proteins: UspA and CspC. The role of the universal stress protein UspA in stress resistance was evaluated by Liu *et al*. [34], who showed that inactivation of the *uspA* gene in *S.* Typhimurium leads to increased susceptibility to stress conditions. A retrospective study reported that the expression of UspA stress protein in *E. coli* was significantly affected by the expression of *cspC* [35]. The *cspC* gene encodes for cold shock protein and is a putative regulator involved in stress response or global gene regulation in *S.* Typhimurium [36]. In addition, Phadtare and co-workers suggested that CspCis one of the important elements involved in the regulation of the expression of UspA and RpoS (a global stress response regulator) [35].

Generally, the metabolic activity in bacteria changes significantly under extreme conditions [37]. Our results showed that two genes encoding for two metabolic enzymes were overexpressed in *S.* Typhimurium: YhhK is a putative acetyl transferase, and GmK is a guanylate kinase. These enzymes have an important contribution in *Salmonella* adaptive response [38]. Additionally, a recent investigation reported that guanylate kinase enzyme has an important role in *E. coli* adaptive response under oxidative stress [39].

In addition to transcription regulator contribution, stress protein accumulation, and metabolic enzyme synthesis, we suggest that the intervention of outer membrane proteins and transport proteins is one of the effective responses to hyperosmotic stress, particularly by importing or producing compatible solutes so that the cells maintain fluid balance and protect cellular proteins from denaturation. Herein, we illustrated that the *yehZ* gene coding for a putative ABC superfamily transport protein was induced in the stressed strain. Recently, the role of YehZ was characterized as an osmoprotectant transporter in *S.* Typhimurium, restraining trehalose production [40]. The *acrA* gene coding for an acridine efflux pump was also induced in the stressed *S.* Typhimurium strain. Accordingly, a previous work described that multidrug efflux pumps have many physiological functions, including transport of drugs, bile salts, toxins, and environmental compounds [41]. We suggest that *S.* Typhimurium utilizes *acrA* efflux pumps induction in response to extracellular signals in order to adapt itself to environmental conditions by transporting salt and environmental compounds. Furthermore, the bacterial envelope stress response is triggered by the accumulation of misfolded outer membrane proteins (OMP) upon envelope damage or excessive OMP synthesis [42]. In accordance with this idea, we found that *stcC* is a gene coding for a putative outer membrane protein and was significantly overexpressed. We believe that StcC plays a crucial rolein outer membrane protein stability and ensuring the adaptability of *S.* Typhimurium during starvation.

S. Typhimurium is characterized by an efficient adaptive response controlled by a set of genes when exposed to extend starvation in salty condition. Previously, Dhiaf and collaborators reported that *S.* Typhimurium was recovered after incubation for 20 years in seawater [8].

DDRT-PCR was selected for the current study because it does not require molecular tools that are currently unavailable. In addition, it is reported to be highly reproducible and very sensitive [20]. Herein, a low number (eight) of 13-Mer arbitrary primers was found to amplify cDNA synthesized from *S.* Typhimurium RNA. We believe that several stress-associated genes were detected, but not all, depending on the selected arbitrary primers set and their amount of guanine and cytosine. This study reinforces the suitability of 13-Mer primers for gene discovery from bacterial cells grown in different conditions. Previously, many modifications of arbitrary primer design have been suggested in order to reduce false positives, simplify amplification, and facilitate cloning and sequencing [43]. The short primers allow frequent annealing of cDNA and are considered optimal to

more accurately represent all mRNA. This is advantageous since few primers are necessary to display most mRNAs. The annealing temperature of the PCR is low to maximize the number of amplified cDNA. However, the higher annealing temperature increases the stringency and reduces false-positive results. Consequently, we propose the application of 40°C in the annealing step when using 13-Mer arbitrary primers to investigate the differential display gene expression in *S.* Typhimurium.

Various methods have been developed to search for differences in expression, but most of them are time or money consuming. Obviously, the DDRT-PCR approach has several advantages over differential hybridization and other molecular strategies used to detect differentially expressed genes. It is a PCR-based method that allows extensive analysis of gene expression among several cell populations; it is faster, sensitive, reproducible and able to identify differentially expressed genes. However, the major limitations of this technique are the false-positive results and the difficulty in confirming differential expression when real-time PCR is not available. In the current study, to avoid false-positive results, our DDRT-PCR analysis was performed independently in triplicate. In addition, given that real-time PCR is not currently available in our lab, the differential expression of the detected genes was confirmed by semi-quantitative RT-PCR using specific primers. DDRT-PCR may be applied in research on bacterial species, particularly to investigate transcriptional profiling; also, this technique may be easier to use in settings with limited access to microarrays [19,44].

Conclusions

Using DDRT-PCR approach, we demonstrated that the transcriptome of *S.* Typhimurium LT2 was altered during one month of starvation in a salty microcosm. Indeed, several genes were shown to be upregulated under these conditions. Faced with salt stress and starvation, *Salmonella* adopted different strategies by modifying the expression levels of a set of genes. Further work may focus on the underlying mechanisms by which environmental stress alters the *Salmonella* virulence expression by examining the individual, as well as the combined contribution, of identified genes in *Salmonella*-host interaction. A future deep characterization of the molecular function of the identified genes may be of great interest to researchers working in the fields of clinical and environmental microbiology. A thorough understanding of *S.* Typhimurium virulence gene

expression in response to environmental stress is critical for the rational design of inhibitors that could prevent the numerous clinical and environmental complications associated with this pathogen.

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Conflict of interests: No conflict of interests is declared.