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Transcriptomic Response of *Salmonella* Typhimurium Heat Shock Gene Expression Under Thermal Stress at 48 °C

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Abstract

Salmonella enterica has been associated with a variety of food products, and thermal treatments are commonly used to reduce or eliminate pathogens from these foods. While the physiological response of *Salmonella* to a wide range of lethal and sublethal heating temperatures has been examined, only limited information is available at the transcriptional level. The objective of this study was to investigate the transcriptional profile of *Salmonella* Typhimurium when subjected to thermal shock at 48 °C (10 min). Transcriptomic analysis was performed using partial microarrays (1152 genes) consisting of quorum sensing, virulence, membrane, and stress related genes. Apparent *Salmonella* viability based on the optical density decreased in response to thermal exposure. Transcriptomic analysis revealed induction of several heat shock and stress related genes due to thermal exposure at 48 °C. This research reveals that there is an impact of exposure to a sublethal temperature (48°C) on the subsequent transcriptional responses of *S.* Typhimurium.

Keywords: *Salmonella* Typhimurium, heat response, microarray, RT-qPCR, gene expression

1. Introduction

Salmonella enterica has been estimated to cause over 1 million foodborne illnesses annually (Scallan et al., 2011), each of which has been estimated to cost $3,221 (based on 2009 currency) (Hoffman, Batz, Morris Jr., 2012; Batz, Hoffman, & Morris Jr., 2014). Additionally, these illnesses often lead to a reduced quality of life due to post-infection complications (Batz et al., 2014). *Salmonella enterica* continues to be linked to foodborne infections, and has previously been associated with a wide variety of food products that are heated at some point during processing, retail, or in the home (Finn, Condell, McClure, Amézquita, & Fanning, 2013; Jackson, Griffin, Cole, Walsh, & Chai, 2013).

In particular, thermal treatment is a common preventative measure taken by food processing facilities, and the appropriate temperature and exposure duration for inactivation is pathogen-dependent (FDA, 2011). It has been suggested that *Salmonella* may become more heat resistant when cells are heated during incremental temperature increases (Mackey & Derrick, 1987), and sublethal heat-shock treatment could potentially cause *Salmonella* to become more virulent and result in more severe illnesses (Mackey & Derrick, 1987a; Humphrey, Richardson, Statton, Rowbury, 1993). For instance, Mackey and Derrick, 1987b found that exposure to 60 °C for 24 minutes resulted in a 10^7-log reduction of *Salmonella* Thompson in reconstituted dried milk, but when cells were exposed to 48 °C for 30 minutes prior to 60 °C treatment, only a 2.4-log reduction was observed. Additionally, Humphrey et al. 1993 reported that when *Salmonella* Enteritidis was exposed to 46 to 48 °C, these cells became more tolerant to heat treatment at 56 °C in comparison to lower pre-exposure temperatures.

Less is known about *Salmonella* transcriptomic responses to thermal exposure (Ricke, Khatiwara, & Kwon, 2013). Milillo et al. (2011) investigated the effects of organic acid and heat exposure at 55 °C on *S.* Typhimurium and observed several differentially expressed genes associated with heat shock following treatment. Additionally,

previous research has focused on *S*. Typhimurium growth response and gene expression responses to heat stress at 42 °C, and transcriptomic analysis of the response to these conditions indicated that several key virulence and fimbrial genes were upregulated (Sirsat, Burkholder, Muthaiyan, Dowd, Bhunia, & Ricke, 2011). While 42 °C was examined as being representative of conditions associated with live poultry body temperature, less is known about higher intermediate non-lethal temperatures that *Salmonella* may become exposed to during post-harvest processing. Further investigation into the growth and transcriptional analysis of *S.* Typhimurium at these temperatures will provide additional insight into how cells respond and tolerate sublethal temperatures. The objective of this study is to use microarray transcriptomic analysis to investigate the effects of heat treatment after exposure at 48 °C for 10 minutes on the expression of *S.* Typhimurium genes that are associated with virulence and heat shock in response to this intermediate sublethal temperature.

2. Materials and Methods

2.1 Growth Conditions and Thermal Treatment

One colony of *S.* Typhimurium ATCC 14028 was inoculated in 5 mL of Luria-Bertani (LB) broth for 18 h incubation at 37 °C. The *S.* Typhimurium ATCC 14028 culture was inoculated in 100 mL LB broth (1%) and incubated at 30 °C to mid-log phase as previously described (Sirsat et al., 2011), and growth measurements were assessed on a spectrophotometer (Spectronic 20D, Milton Roy Company, Rochester, NY, USA) at OD_{600} over 24 h. A 1 mL aliquot of a mid-log (approximately 0.4 OD_{600}) S. Typhimurium culture was centrifuged (6,000 × g for 10 min) and prepared for transcriptional microarray by resuspension in 2 mL phosphate buffered saline (pH 7.4 PBS). Thermal stress was applied to bacterial cells for 10 min in a 48°C water bath.

2.2 Microarray Design

A microarray chip embedded with 43 to 45-mers oligonucleotides was designed for transcriptomic analysis of *S.* Typhimurium LT2 (McClelland et al., 2001). A total of 1152 genes out of nearly 6000 currently available were chosen for microarray analysis. These genes were chosen due to their association with virulence, membrane, stress, quorum sensing and transcriptional regulation for evaluation of gene expression under thermal stress at 48 °C. Oligos were synthesized, normalized, resuspended, and printed as previously described (Sirsat et al., 2011). Information for the array format had previously been submitted (accession number GPL9181) (Sirsat et al., 2011) in the NCBI Gene Expression Omnibus (GEO) (Edgar, Domrachev, & Lash, 2002).

2.3 RNA Extraction and Probe Synthesis

Three biological replicates of RNA extraction and probe synthesis were performed as previously described (Sirsat et al., 2011). Briefly, cells were exposed to 48 °C for 10 min, resuspended in RNAprotect Bacteria Reagent (Qiagen, Valencia, CA, USA), and the RNeasy Bacteria Mini Kit (Qiagen Inc.) was used to extract total RNA. After treating with RNase-free DNase (Qiagen) to remove residual DNA, RNA quality and quantity was assessed with a sprectrophotometer. Following reverse transcription, the cDNA was subsequently labeled and hybridized to the microarray as previously described (Sirsat et al., 2011).

2.4 Microarray Analysis

A Genepix 4000B (Molecular Devices Corporation, Union City, CA, USA) was used to scan microarray images followed by analysis with GenePix 6.0 software (Molecular Devices Coporation). Matrix Laboratory (MATLAB) 2009 software and Microsoft Excel were used for statistical analyses. Logically weighted scatterplot smoothing (LOWESS) normalization was used to remove any dye and/or systematic biases (Sirsat et al., 2011; Quackenbush, 2002), and additional data analysis was conducted using LOWESS normalized log ratio values. A student's t-test was used to generate P-values from normalized log ratio values after correcting for a false discover rate (FDR less than 0.05) (Benjamini & Hochberg, 1995).

Genes that exhibited at least a 2.0 fold up or down regulation after the FDR correction were included in the final data set. The functional categories used to classify genes were derived from previous research data sets generated by Sirsat et al., (2011) using the comprehensive microbial resource of TIGR (http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi), as well as the KEGG GENOME resource (http://www.genome.jp/kegg-bin/show_organism?org=T00065). This data is accessible via series accession number GSE18089 after having been deposited in NCBI's GEO (Edgar et al., 2002), and can be accessed through series accession number GSE18089 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18089) (Sirsat et al., 2011).

2.5 Real-time Quantitative RT-PCR (qRT-PCR)

For the real time quantitative RT-PCR (qRT-PCR) assay, specific primer pairs were constructed for *dnaK*, *ibpA*, *uspA*, *fliL*, and *flgF* genes to validate the microarray data. The RNA harvested from control and thermally stressed (48 °C) microbial populations were analyzed using ABI 7500 Sequence Detection system (PE Applied Biosystems, Foster City, CA, USA) and QuantiTect SYBER Green RT-PCR. The relative induction or repression of genes was reported via the $2^{-\Delta\Delta Ct}$ method using the 16S rRNA as a reference gene to normalize cycle threshold values (Livak & Schmittgen, 2001; Dowd, Killinger-Mann, Blanton, San Francisco, & Brashears, 2007).

3. Results and Discussion

To investigate the effects of thermal stress at 48 °C on the growth response of *S.* Typhimurium, populations of mid-log phase *S.* Typhimurium were measured (OD_{600}) after transferring to a 48 °C water bath. The optical density (OD_{600}) measurements indicate that thermal stress at 48°C caused a decrease in *Salmonella* viability similar to previous work (Sirsat et al., 2011) with the organism (Figure 1). The average OD_{600} values (average ± standard deviation) of *S.* Typhimurium grown at 48 °C were slightly lower after 6, 11, and 24 h of growth (0.33 ± 0, 0.03 ± 0, and 0.30 ± 0.01), respectively) when compared to previous work by Sirsat et al. (2011). In the previous research, *S.* Typhimurium was grown at 30 °C, and after 6, 11, and 24 h of growth (0.38 ± 0.01, 0.43 ± 0, and 0.45 ± 0.01, respectively), these cultures exhibited OD_{600} values similar to the *S.* Typhimurium cells at 42 °C.

The effects of thermal stress (48 °C) on gene expression in *S.* Typhimurium was determined by subjecting a mid-log cell population to 48 °C for 10 min, and performing transcriptomic analysis using a microarray. The ten-minute treatment provided a means of comparison with previous work by Sirsat et al. (2011). A microarray chip used in previous research (Sirsat et al., 2011) that consisted of 1152 genes from virulence, membrane, stress, quorum sensing, and transcriptional regulation functional groups was evaluated. The induced and repressed *Salmonella* genes in response to thermal stress at 48°C are available in supplemental files 2 and 3, respectively.

Comparison among microarray and qRT-PCR revealed some differences between these two methods with regard to gene expression for 48 °C. For instance, *dnaK* was determined to be upregulated 23-fold, while qRT-PCR indicated that *dnaK* was upregulated 44-fold (Table 1). Additionally, *fliL* and *flgF* genes were repressed roughly 20-fold as indicated by microarray, but minimal changes in gene expression were observed with qRT-PCR (Table 1). Sirsat et al. (2011) also observed similar inconsistencies between these two methods. The particular system being investigated factors into how well qRT-PCR data supports microarray analysis, and it has been suggested qRT-PCR data is supportive of microarray results roughly 70% of the time (Ding et al., 2007). Smaller differences in gene expression are typically observed with qRT-PCR in comparison to microarray data, and it is often difficult to obtain similar results with different technology platforms (Ding et al., 2007; Sinicropi et al., 2007; Wang et al., 2006).

Table 1. Comparison of microarray and qRT-PCR data

Locus ID	Gene	Gene-Protein name	Microarray [a]	qRT-PCR [a]
AAL18976.1	*dnaK*	Chaperone Hsp70	23.38	44.04
AAL22668.1	*ibpA*	Small heat shock protein	16.47	16.56
AAL22451.1	*uspA*	Universal stress protein A	3.21	1.92
AAL20887.1	*fliL*	Flagellar biosynthesis	-22.48	1.29
AAL20108.1	*flgF*	Flagella biosynthesis protein	-18.07	-0.12

[a]All values represent fold-change.

The microarray transcriptomic analysis revealed induction of several heat shock related genes (Table 2). Both *dnaK* and *dnaJ* encode chaperones that are involved in several cellular processes including binding to misfolded proteins, which allows sigma H (encoded by *rpoH*) to direct the transcription of heat shock proteins (Spector & Kenyon, 2012). Additionally, the DnaK-DnaJ complex has been suggested to enhance survival of *Salmonella* in macrophages (Takaya, Tomoyasu, Matsui, & Yamamoto, 2014). Given that heat shock response is transient, it follows that several stress factors including genes in the Rpo regulon *(rpoS, rpoE*, and *rpoH*) would be upregulated after being exposed to 48 °C.

Table 2. Expression fold value of virulence-related genes in thermal-stressed *Salmonella*

Locus ID	Gene symbol	Gene/Protein name	48°C for 10 min
AAL22428.1	*rpoH*	Sigma H factor of RNA polymerase	11.16
AAL20321.1	*sseA*	Secretion system effector	7.25
AAL19173.1	*htrA*	Periplasmic serine protease	4.11
AAL20322.1	*sseB*	Secretion system effector	3.46
AAL21534.1	*rpoE*	Sigma E (sigma 24) factor of RNA polymerase	3.11
AAL21804.1	*rpoS*	Sigma S (sigma 38) factor of RNA polymerase	2.75
AAL20341.1	*ssaP*	Secretion system apparatus protein	2.32
AAL21778.1	*invG*	Outer membrane invasion protein	-3.01
AAL21754.1	*prgH*	Cell invasion protein	-3.74
AAL21774.1	*invC*	Surface presentation of antigens	-4.89
AAL21751.1	*prgK*	Cell invasion protein	-5.48

As expected, these genes were induced slightly more at 48 °C (*rpoS* (2.75), *rpoE* (3.11), and *rpoH* (11.16)) when compared to the gene expression of cells exposed to heat stress at 42°C (*rpoS* (2.58), *rpoE* (2.2), and *rpoH* (7.61)) in our previous research (Sirsat et al., 2011). However, it should be noted that the transcription levels of *rpoH* are considered to be mostly independent of temperature fluctuations, and that the concentration of sigma H is regulated at the translation level (Spector & Kenyon, 2012). RpoS is associated with RNA polymerase and controls the expression of up to 50 *Salmonella* proteins (Humphrey, 2004). RpoS is produced in response to starvation, as well as pH and temperature fluctuations (Humphrey, 2004; Lianou & Koutsoumanis, 2013).

Food products that are temperature abused may possibly cause *Salmonella* cells to be more virulent. The changes in gene expression observed in this particular study could be used as a preliminary finding to track these changes over time while a food product is being thermally processed. Interestingly, Millilo et al. (2011) observed repression of several heat shock-related genes following exposure to both sodium acetate and sodium propionate at 55 °C. However, the transcriptional response to these conditions likely differ from an independent analysis of gene responses to 55 °C, which would be necessary to determine these responses to various heat treatments. Previous studies indicate that when *Salmonella* had been exposed to 48 °C, cells were still heat resistance for up to 10 hours in temperatures ranging from 50 to 59 °C (Mackey & Derrick, 1986). *Salmonella* may respond to sublethal heat differently depending on the absolute temperature(s) in which they are exposed. To address this question, *Salmonella* could be transitionally exposed to a range of specific sublethal temperatures over time followed by an assessment of transcriptional responses. Additionally, future research may need to focus on the expression of *Salmonella* genes over an extended period of time after the exposure and return to a more optimal temperature. A time-course gene expression study would be useful to indicate the potential risk heat shocked *Salmonella* cells could pose once the food they harbor is being consumed. As suggested by Millilo et al. (2011), additional studies on the transcriptional profile of *Salmonella* would help guide effective multiple hurdle approaches for *Salmonella* at lower thermal temperatures. Additionally, future research can implement RNA sequencing procedures as a means to validate quantitative PCR data as well as avoid the use of microarray techniques that can pose questionable results.

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