Chapter I

Analyzing the effect of external heat induction in the immunity and longevity of *Caenorhabditis elegans*
Stressors have a major influence on the human body and involves in modulation of internal homeostasis. Stressors can be of various sources namely physical, chemical or biological. Heat stress, oxidative stress, hypoxia and osmotic stress are classified under physical stressors that impacts greatly on the longevity of each individual. Biological stress such as pathogenic exposure also determines the status of immune system and the responses elicited against it. Besides, physical stressors are either considered beneficial or unfavourable to the human. Among them, heat stress plays a pivotal role in maintaining and activating important regulatory players of higher invertebrates. Heat stress was earlier classified as harmful stressor, however limited or optimum heat for a short period of time can turn favourable to the host system. During thermal stress, certain set of proteins called heat shock proteins (HSPs) are modulated and serve as molecular chaperones to prevent protein aggregation and helps transport of repaired proteins (Iguchi et al., 2012). HSPs, which invokes upon heat stress have gained much prominence in aging research and are thought to be of importance for both longevity and overall maintenance of proteome integrity with advancing age. The most compelling evidence so far has been collected from invertebrate models such as Drosophila and Zebra fish, in which, joint effects of longevity and thermo tolerance were found to be the plausible reason for the increased lifespan of these models (Lithgow et al., 1994 & 1995). The conserved function of HSPs across species has made the research divert towards the heat shock response that relates to the lifespan, healthspan, and disease pathology.

*C. elegans* serves as an imperative model for human diseases since it has many common biological features with humans, such as the development of muscles, digestive tract and nerves (Markaki and Tavernarakis, 2010). However, *C. elegans*, a simple soil nematode, which is considered as a versatile model for aging studies, has been exploited very less for heat stress related lifespan extension against bacterial pathogens. HSPs are considered as
quintessential molecules produced under heat stress and certain external stresses in this nematode (Kono and Rock, 2008; Martin-Murphy et al., 2010). One of the noteworthy characteristics of HSPs is their immuno dominance. However, the fallouts of these molecular chaperones are under the control of Heat Shock Factor-1 (HSF-1). The heat-shock response has been implicated to work through three neuroendocrine signaling pathways namely: the nuclear hormone receptor (NR) pathway, the transforming growth factor-β (TGF-b) pathway, and the IGF/insulin-like signaling (IIS) pathway (Rodriguez et al., 2013). Both HSF-1 and DAF-16 are negatively regulated by IIS pathway’s sole receptor DAF-2. Activation of DAF-2 results in sequential phosphorylation events thereby preventing DAF-16 to act on the target gene networks (Henderson and Johnson, 2001). Similarly, HSF-1 cellular localization is controlled by a HSF-1-interacting protein DDL-1 (Chiang et al., 2012). The stimulation of HSF-1 during heat stress undergoes a hierarchical process starting from oligomerization, post-translational modification, and nuclear translocation. These serial events altogether end up in a synergistic effect of HSPs (Barna et al., 2012). The DNA-protein interaction in *D. melanogaster* is the first study to reveal that HSF binds to the heat shock element (HSE) to induce HSPs (Parker and Topol, 1984). Numerous reports have been published that support the statement that attenuation of IIS pathway activates HSF-1 (Hsu et al., 2003; Morley and Morimoto, 2004). Mutation in *daf-2* gene, which is principal component of IIS pathway, also leads to similar kind of phenomenon observed in HSF-1 (Halascheck-Wiener et al., 2005). Moreover, IIS regulation has also been reported to stimulate the response of *C. elegans* towards a wide range of pathogenic bacteria (Fontana et al., 2010; Kenyon, 2010). Enhanced pathogen resistance was observed in *daf-2* mutants during *P. aeruginosa* infection while *daf-16* mutants were short lived (Evans et al., 2008). Upon heat stress, transcription and translation of most proteins are inhibited. HSPs expression is thought to have roles in development and heat stress response by folding of polypeptides in the course of synthesis,
prevents protein denaturation or aggregation and guides proteins for degradation when refolding is unsuccessful (Hartl and Hayer-Hartl, 2002; Chang et al., 2007).

Even though the HSPs role is conserved among different species, there is a cell to cell variation in the expression during aging (Soti et al., 2005; Gamerdinger et al., 2009). But their total capacity and regulation upon external heat shock and consecutive infection are still not under the limelight. HSF-1 is inhibited by a negative feedback loop mediated by the certain HSPs, while activation of HSF-1occurs when equilibrium of chaperones shifts toward association with metastable polypeptides (Guisbert et al., 2013).

Mild heat stress impinge activation of certain stress responses could be sufficient to extend life span (Lithgow et al., 1995; Cypser and Johnson, 2002). With this background, the current study was taken to examine the outcome of heat shock response and its role in lengthening the lifespan during bacterial encounter. Employing the nematodes, key aspects of the heat shock response have been well established at a cellular level, yet the heat shock response has additional features that show culture-specific differences in chaperoning in the regulation of the HSF-1 which was analysed with an external induction of heat. The link between the IIS pathway and expression of heat shock genes/ proteins in presence of pathogens was explored and our observations impart evidence that HSF-1 confers longevity to C. elegans by enhancing the immunity to certain magnitude.

MATERIALS AND METHODS

Media

**Nematode Growth Medium (NGM)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>3 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>3 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
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</table>
The content was autoclaved for 50 min at 121°C

1 M KPO$_4$ buffer pH 6.0 25 mL
1 M CaCl$_2$ 1 mL
1 M MgSO$_4$ 1 mL
Cholesterol (dissolved in 95 % ethanol in a concentration of 5 mg/mL) 1 mL
Streptomycin (1 mg/mL) 100 μL
Nystatin (200 mg/mL) 100 μL

**Luria Bertani (LB)**

- Tryptone 10 g
- Yeast extract 5 g
- NaCl 10 g
- pH $7.0 \pm 0.2$
- Agar (Bacteriological grade, HiMedia) 1.8 %

**Nutrient Agar**

- Peptone 10 g
- NaCl 10 g
- Yeast Extract 5 g
- pH $6.8 \pm 0.2$
- Agar (Bacteriological grade, HiMedia) 1.8 %

**Antibiotic stock solution**

**Streptomycin** (1 mg/mL)

1 mg of streptomycin sulphate salt was dissolved in 1 mL of sterile distilled and stored at 4 °C.
Nystatin (200 mg/mL)

In 1 mL of dimethyl sulfoxide (DMSO), 200 mg of nystatin was dissolved and stored at -20 °C.

- **Buffers**

  **M9 Buffer**
  
  KH$_2$PO$_4$  
  Na$_2$HPO$_4$  
  NaCl  
  MgSO$_4$7H$_2$O  

  **S Buffer**
  
  0.05 M K$_2$HPO$_4$  
  0.05 M KH$_2$PO$_4$  
  NaCl

  **Bleaching Solution**

  An equal ratio (1:1) of 5 M KOH and household bleach (5 % solution of sodium hypochlorite) was freshly prepared for the synchronization of nematodes.

- **Solutions for protein estimation**

  **Protein Standard: (Bovine Serum Albumin –BSA)**

  Stock – 1mg/ml (1mg of BSA dissolved in 1ml of deionized water)

  Working solution- 100 µl of 1 mg/ml + 900 µl of deionized water

  **Solutions for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

  **10% SDS**

  SDS 10 g

  Dissolved in 100 ml of MQ water
1.5 M Tris-HCl, pH 8.8

- Tris: 18.17 g
- Deionized water: 75 ml

pH adjusted to 8.8 with conc. HCl. Volume made up with MQ water to 100 ml

Stored at 4°C

0.5 M Tris-HCl, pH 6.8

- Tris: 6.05 g
- Deionized water: 75 ml

pH adjusted to 6.8 with conc. HCl. Volume made up with double distilled H2O water to 100 ml

Stored at 4°C

5X Sample buffer

- 1 M Tris-HCl pH 6.8: 0.6 ml
- 50 % glycerol: 5 ml
- 10 % SDS: 2 ml
- 10 % β-mercaptoethanol: 1 ml
- 1 % bromophenol blue: 0.5 ml

Volume made up with deionized water to 10 ml

Aliquots (1 ml) were stored at -20°C

30% Acrylamide

- Acrylamide: 29 g
- Bis-acrylamide: 1 g

Volume made up with deionized water to 100 ml

Aliquots (1 ml) were stored at 4°C.
**10X Tris-glycine buffer** (tank buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14 g</td>
</tr>
<tr>
<td>SDS</td>
<td>2.5 g</td>
</tr>
<tr>
<td>pH</td>
<td>8.3</td>
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</table>

Volume made up with deionized water to 100 ml

**10% APS (Ammonium per sulfate)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>10 g</td>
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</tbody>
</table>

Dissolved in 100 ml of deionized water

**Resolving Gel (12 %)**

<table>
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<tr>
<td>Distilled water</td>
<td>6.6 ml</td>
</tr>
<tr>
<td>30 % Acrylamide mix</td>
<td>8 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>5 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.008 ml</td>
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</table>

**Stacking Gel (5 %)**

<table>
<thead>
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<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>6.8 ml</td>
</tr>
<tr>
<td>30 % Acrylamide mix</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>1M Tris (pH 6.8)</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.14 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

❖ **Solutions for Western Blot**

**Blocking Buffer (5 %)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder (fat free)</td>
<td>5 gm</td>
</tr>
</tbody>
</table>

Make up to 100 ml with TBST buffer.

**Transfer Buffer:**

1. **Anode I Buffer (50 ml):**
(Working Conc: 300 mM Tris (pH 10.4), 20 % Methanol)

1M Tris pH 10.4  
Methanol +  
DD water

25 ml

2. **Anode II Buffer (50 ml):**

(Working Conc: 25 mM Tris (pH10.4), 20 % Methanol)

1M Tris (pH 10.4)  
Methanol +  
DD water

1.25 ml  
10 ml  
38.75 ml

3. **Cathode buffer (100 ml)**

(Working Conc: 25 mM Tris (pH 9.4), 20 % Methanol)

1M Tris (pH 9.4)  
Methanol  
DD water

2.5 ml  
10 ml  
77.5 ml

**TBS- Buffer:** (Prepare in 10X concentration)

Working concentration (1X)

Tris base-(pH-7.5)  
Sodium chloride

10 mM  
150 mM

**TBS-T:** (final concentration of tween-20 is 0.1 %)

999ml of 10X TBS was taken and to that 1 ml of tween-20 was added

**Primary Antibody:** (can reuse for 3-4 times)

1:200 dilution in 3% of blocking buffer

**3% blocking buffer-:**

5% blocking buffer  
1XTBST

6 ml  
4 ml

**Primary antibody mix (10 ml):**

Primary antibody  
3% blocking buffer

50µl  
9950 µl

**Secondary Antibody:** (freshly prepared)

1: 1000 dilutions in 2.5 % blocking buffer

Preparation of 2.5 % blocking buffer:

5% blocking buffer  
1X TBST

5 ml  
5 ml
Secondary antibody mix (10 ml):

- Secondary antibody 10 µl
- 2.5 % blocking buffer 990 µl

**BCIP**

50 mg/ml or 12.5 mg/250ul (prepared in autoclaved double distilled (DD) water)

Note: easily get oxidised and turns to blue colour; BCIP if it’s oxidised was not used.

**NBT**

10 mg/ml (prepared in autoclaved DD water)

**5X AP Buffer:**

Stock was prepared in 5X concentration

Working concentration (1X)

- Tris-base (pH 7.5) 100 mM
- Sodium chloride 100 mM
- Magnesium chloride 100 mM

**Developer (10 ml):**

Prepare 1X of AP buffer for 10 ml

(2 ml of 5X AP buffer + 8ml of DD Water)

Add 1.65 µg of NBT + 33µg/ml of BCIP

**Maintenance of *C. elegans***

The wild type (N2 Bristol) and mutant strains used in the present study were obtained from *Caenorhabditis* Genetics Center (CGC), funded by NIH National Center for Research Resource, Minnesota, USA and maintained in the laboratory by following standard procedures given below.
Preparation of NGM plates

The *C. elegans* were maintained and grown in NGM plate seeded with *Escherichia coli* OP50, a laboratory food source *(Brenner, 1974)*. *E. coli* OP50 being a uracil auxotroph, shows limited growth with a translucent colony in NGM agar. The medium was prepared in a sterile petri-dish of different sizes. According to the nature of experiment, the size of the NGM plates varies. Approximately 0.2 mL to 0.5 mL of liquid culture of *E. coli* OP50 was placed on the NGM plate using pipet. The culture was spread by gently tilting the plates and care was taken that the lawn did not touch the medium and wall interface of the plate. Finally, the plates were incubated at 37 °C for 8 h to get a lawn of *E. coli* OP50 and the plates were stored in an air-tight container at 4 °C and taken for use within 2-3 weeks.

Transferring nematodes to NGM plates

Transfer of nematodes was performed by several methods such as chunking, picking nematodes by platinum loop worm picker or by strips of filter paper. Among these, chunking was faster and easier method to maintain homozygous stocks. The agar containing nematodes were chunked using sterile scalpel and transferred to a new NGM plate. The nematodes present in the chunked agar migrated to the fresh lawn of *E. coli* in new NGM plate. The nematodes were visualized under dissecting binocular stereo zoom microscope equipped with a transmitted light source with a standard 10X eyepieces *(Nikon SMZ1000, Japan)* and objectives ranges from 0.8X to 8X.

Freezing and recovery of nematode stock

Storage and revival of stocks make *C. elegans* one of ideal models to in the research field. The stocks can be frozen and revived whenever needed. Liquid freezing and soft agar freezing are the two methods followed. For both the methods, freshly starved L1 –L2 stage
nematodes were used. In the long term storage, liquid nitrogen was used to freeze the nematode stocks. In this method, the nematodes get settled at the bottom of the vial; hence the whole content of the container was transferred to the NGM plate. Whereas, in soft agar method the supernatant is taken since the nematodes are suspended in the upper phase and glycerol and S buffer settles at the bottom. The cyrovials were labelled with strain name, date and stored at -80°C deep-freezer for further use. During revival, the vial stored at -80°C was taken and a scoop of soft-agar was transferred to the NGM plates seeded with *E. coli* OP50. The percentage of worm recovery was more when compared to the liquid freezing method.

**Bacterial strains and culture condition**

*S. aureus* (No. 11632) and *E. coli* OP50 were maintained in Luria Bertani medium, while *P. mirabilis* (No. 7002) was maintained in nutrient medium. *S. aureus* and *P. mirabilis* were obtained from American type culture collection (ATCC, USA) and were maintained at 37 °C with aeration. *E. coli* OP50 was obtained from CGC was also maintained at 37 °C. All assays were performed with a constant bacterial inoculum (0.3 OD at 600 nm), i.e 12 x 10^6 cells/ml for *P. mirabilis* and 18 x 10^6 cells/ml for *S. aureus*.

**Synchronization of *C. elegans***

The wild type and mutant *C. elegans* were maintained routinely at an optimum temperature of 20 °C and 15 °C respectively, on NGM seeded with *E. coli* OP50. The strains used in this chapter were *C. elegans* WT Bristol N2, *daf-2* mutant (*e1370*), *hsf-1* mutant (*sy441*) and *daf-21* mutants [JT6130 (p673)V and PR673(p673)V]. Age-synchronized worms were obtained by treating the gravid nematodes with commercial bleach (hypochlorite) solution containing 5 M potassium hydroxide in 1:1 ratio. The tube was vortexed for 2 min and the mixture was diluted with M9 buffer. The tube containing the nematode suspension
was centrifuged for 1 min at 1000 rpm to pellet the released eggs. The supernatant was discarded and the eggs were transferred to the edge of the NGM plate seeded with an *E. coli* OP50 lawn (*Sulston 1983*). These stage-synchronized L4 worms were used in all assays.

**Nematode lifespan assay**

Lifespan assays were performed using stage (L4) synchronized population of nematodes. Synchronization was attained by bleaching the nematodes. The synchronized population was divided into two sets; initially one set was provided with a heat shock for 2 h at 35 °C with a subsequent recovery period for 4 h at 20 °C, wherein the other set was continuously maintained at 20 °C. Twenty numbers of synchronized L4 stage *C. elegans daf-2* mutant, *hsf-1* mutant, *daf-21* mutants from both heat shock induced and un-induced plates were exposed to the pathogens (in individual wells), *P. mirabilis* and *S. aureus* contained in 24 well microtiter plates. The nematodes (heat shock induced and un-induced) exposed to laboratory food source *E. coli* OP50 was taken as control. The microtiter plates were incubated at 20 °C and the life-span changes were examined for every 6 h throughout the assay period. The nematode that does not respond when disturbed with a platinum wire or ceased the pharyngeal pumping was scored as dead.

**Chemotaxis assay**

The difference in the food preference of external heat shock induced and un-induced wild type nematode was assessed by chemotaxis assay. In Brief, cultures of pathogen (either *P. mirabilis* or *S. aureus* (zone A)) and *E. coli* OP50 (zone B) were spotted at a distance of 3 cm from the centre of NGM plates (90 mm). Subsequently, twenty five wild type nematodes from each set (heat shock induced and un-induced) of populations were thoroughly washed from *E. coli* OP50 lawn and placed at the centre of the plate and the preference of the nematode was monitored. Total number of nematodes that have moved towards each zone
was counted every 4 h and the results were plotted with 12 h interval up to 48 h. The assay controls were included in which both zone A and B were spotted with OP50 or *P. mirabilis* or *S. aureus*.

**Microscopic observations**

For microscopic visualization, external heat induced and un-induced nematodes were infected with *P. mirabilis* and *S. aureus* individually for a time period of 12, 24 and 36 h. The infected nematodes were washed thrice with M9 buffer and placed in a 1 mM sodium azide solution containing 2 % agarose pad for microscopic observations under inverted fluorescent microscope (Nikon, Japan). The phenotypic changes were monitored and recorded using an attached digital camera.

**Total RNA isolation and real-time PCR analysis**

Quantitative PCR assay was performed with synchronized nematodes (L4 stage). The bacteria adhered to the surface of nematodes (control and treated samples) were washed with M9 buffer and flash frozen. Total RNA was isolated using Trizol method (*Morse and Bass, 1999*). The isolated RNA was converted into cDNA using kit (High capacity cDNA Reverse Transcription kit, Applied Biosystems). Quantitative PCR was performed in a single well format using sterile 96-well plates as per the manufacturer’s instructions. The *C. elegans* gene-specific primers and primers for house-keeping gene (β-actin) were combined individually with qPCR mix (SYBR Green kit, Applied Biosystems) at a predefined ratio. The qPCR cycle numbers were titrated as per the manufacturer’s protocol to ensure that the reaction was monitored and recorded by covering the linear range of amplification. The steady-state levels of candidate gene mRNA were assessed from the cycle threshold (Ct) values relative to the Ct values of the internal control β-actin mRNA of the same samples. The expression levels of candidate genes were normalized by β-actin (*act-2*). Gene
expression was measured by calculating $2^{-\Delta \Delta Ct}$. The lists of primers used are mentioned in Table 1.1.

**Table 1.1.** List of primer sequences used in the present study

<table>
<thead>
<tr>
<th>S.no</th>
<th>Gene name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β- actin</td>
<td>ATCGTCCTCGACTCTGGAGATG</td>
<td>ACGTCCAGCCAAAGTCAAG</td>
</tr>
<tr>
<td>2</td>
<td>tol-1</td>
<td>CGTCAATGAGCTTCCATCA</td>
<td>ATGGGCTTCAGCATCAGGTT</td>
</tr>
<tr>
<td>3</td>
<td>hsp-1</td>
<td>AGCTCTGCCTGATCCTCCAT</td>
<td>TCCTGGACAGCCTCAGACTT</td>
</tr>
<tr>
<td>4</td>
<td>hsp-6</td>
<td>CCCTTACAACTCTGCAAAAT</td>
<td>GCTCGTTGATGACACGAA</td>
</tr>
<tr>
<td>5</td>
<td>bec-1</td>
<td>TCGCTGAAGCCGACGCCTCA</td>
<td>ACTACCACATGCGACGCGGCA</td>
</tr>
<tr>
<td>6</td>
<td>daf-21</td>
<td>CATTCCAGGCTGAGATTGCT</td>
<td>GTCACAGACTTGGCAATGGT</td>
</tr>
<tr>
<td>7</td>
<td>age-1</td>
<td>ATAGAGCTCCACGGCAATTT</td>
<td>TGTCAAGCACTTTCTTTCTCG</td>
</tr>
<tr>
<td>8</td>
<td>daf-2</td>
<td>TCGAGCTTCCCTACGGGCT</td>
<td>CATCTTGTCACCAAGTGATC</td>
</tr>
<tr>
<td>9</td>
<td>daf-16</td>
<td>TGGTGGAATTCAATCGTGAA</td>
<td>ATGAATATGCTGC CCTCCAG</td>
</tr>
<tr>
<td>10</td>
<td>sgk-1</td>
<td>TCAGGCACAAGGAGACCTAAA</td>
<td>GTGAAACAAAGGAAAGGTG</td>
</tr>
</tbody>
</table>

**Western blotting**

Independent total protein samples were isolated from wild type and daf-2 mutant nematodes induced with heat shock and subsequently exposed to pathogen as detailed in the nematode lifespan assay. For control, total protein isolated from nematodes exposed to *E. coli* OP50 was taken. Protein extraction was done by subjecting the worm aliquots to sonication and quantified using Bradford’s method. Prior to sonication, protease inhibitor cocktail (Sigma) was added to each sample to prevent protein degradation. The separation of proteins was carried out with 12 % SDS-PAGE. Soon after the separation, the gel was equilibrated in cathode transfer buffer for 30 minutes. A Polyvinylidenedifluoride (PVDF)
membrane was cut as the same size of gel and soaked in methanol. The protein was transferred to a PVDF membrane [Whatman, GE Healthcare] using semi-dry blotting apparatus (Amersham, UK) (Durai et al., 2014). Blotting was carried out at constant voltage of 15V for 3 hours. After blotting, the membrane was transferred into blocking buffer and incubated at 4 °C in rocker overnight. Consequently, the membrane carrying the transferred proteins was incubated in blocking solution of 5% defatted skim milk powder (HiMedia Laboratories, India) dissolved in Tris-buffered saline containing 0.1 % Tween-20 for 3 h at 4 °C. The membrane was probed individually with Anti- HSF-1, anti- HSP-90, anti- SGK-1 antibody and also with anti β- ACTIN as a loading control for 6 h at 4 °C and then in the secondary antibody conjugated with Alkaline Phosphatase (AP) enzyme for 4 h at 4 °C. The membrane was subsequently taken for immuno detection by developing it in 1X AP buffer solution containing the substrates nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolylphosphate (BCIP) and left until band appears. By using ImageLab software the quantification and fold change were determined.

**Statistical analysis**

The experiments carried out in this study were performed in triplicates. Statistical significance of choice index between control and pathogen groups were analyzed one-way ANOVA (SPSS 17). The average of three independent experiments’ data were taken and the significance in differences between the means of different parameters were determined using Duncan followed by Dunnet’s test ($p<0.05$) comparing between the groups (Control Vs Treated).
Results

Heat shock induction increases longevity of nematode against *S. aureus*

The impact of external heat induction on the lifespan of wild type (N2) *C. elegans* was checked by exposing the nematodes to a Gram positive pathogen, *S. aureus* and a Gram negative opportunistic pathogen, *P. mirabilis* with and without heat induction prior to exposure. Complete killing of nematodes was achieved at $90 \pm 2$ h in the heat un-induced samples that was exposed to *S. aureus*, while, the nematodes induced with external heat stress before *S. aureus* infection showed extended lifespan up to $102 \pm 2$ h (Fig 1.1). Heat induction earlier to pathogenic exposure lengthens the survival rate of nematode to certain extent. Similarly, the same experiment was performed against opportunistic pathogen; *P. mirabilis* and the laboratory food source *E. coli* OP50. The survival of N2 exposed to both the strains was not significantly different between the heat shock induced and un-induced groups.

![figure 1.1](image.png)

**Figure 1.1.** Survival of wild type nematode pre exposed to heat shock and subsequently infected with *S. aureus* and *P. mirabilis*. Nematodes fed on *E. coli* OP50 were considered as control.
Food preference of nematode does not alter with heat induction

Furthermore, this observation led to evaluate the preference of *C. elegans* towards food over pathogen for which chemotaxis assay was performed. The nematodes were found to be attracted more towards *P. mirabilis* than to *E. coli* OP50 (Fig 1.2 A). Conversely, both the heat induced and un-induced nematodes moved towards the food source, showing aversion towards *S. aureus* in the *S. aureus* spotted plates (Fig 1.2 B). However, no difference in the preference was observed between heat induced and un-induced nematodes.

![Figure 1.2. Food preference of the nematode after external heat induction (A) against *P. mirabilis* (P.m) (B) against *S. aureus* (S.a).](image)

External heat induction reduced the intestinal colonization

A deeper insight on physiological changes in nematodes during heat induction and consecutive pathogenic exposure was visualized by microscopic examination. In the control and *P. mirabilis* exposed nematodes there was no significant change, strikingly, prominent changes were observed in *S. aureus* exposed nematodes. The heat shock induced nematodes showed a decreased amount of colonization, wherein, un-induced group had an elevated amount of colonization. Moreover, the nematodes which were not influenced with heat stress showed pharyngeal grinder disruption and immature eggs in the later hours (Fig 1.3).
Elevated levels of *daf-16* and *sgk-1* increases the nematode lifespan

Since the heat induction involves in extension of lifespan, the regulation of *daf-2*, *age-1*, *daf-16*, *sgk-1* were assessed. The above mentioned genes take part in insulin signaling (IIS) pathway. In the present study, no significant regulation of *daf-2* was observed in both induced and un-induced nematodes during *P. mirabilis* exposure. Inversely, a substantial down regulation in the expression of *daf-2* was detected in heat induced samples during *S. aureus* exposure. In addition, no significant modulation in expression was observed for *age-1* in both heat induced and un-induced groups with *P. mirabilis* and *S. aureus* exposure except for 24 h exposed samples (Fig 1.4A and B). Report by Kenyon et al, 1993 states that, down regulation of *daf-2* prevent activation of *age-1*; directing *daf-16* for nuclear translocation. This intended the present study to look for the expression of *daf-16*, which in current study was observed to be upregulated at 12 h when compared to its expression in the 24 and 36 h in heat induced samples and in the un-induced groups during *P. mirabilis* exposure (Fig 1.4C).
In the nematodes that were exposed to *S. aureus* the heat induced samples had considerable upregulation of *daf-16* but was down regulated in the un-induced samples as the exposure time increased (Fig 1.4D). The expression levels of *sgk-1*(Gatsi et al., 2014), a serum glucocorticoid inducible kinase exhibited steady up regulation in all the time points of heat induced samples subsequently exposed to *P. mirabilis* (Fig 1.4C). On the other hand, N2 exposed to *S. aureus* had a decline in regulation at 36 h in the heat induced samples (Fig 1.4D).

The genes (*hsp-1, hsp-6, hsp-90*) were monitored for its regulation during external heat induction (Chiang et al., 2012). Markedly, *hsp-1* and *hsp-6* were observed to be up regulated in both *S. aureus* and *P. mirabilis* exposed nematodes at the initial hours of heat induction (12 h). Reduction of *daf-21* levels in wild type nematodes leads to reduced motility and induction of the muscular stress response. In contrast, in the heat shock un-induced samples, all the heat shock responsive genes were down regulated except *daf-21* at 24 and 36 h (Fig 1.4E and F).

The expression level of genes (*tol-1, bec-1*) representing different immune pathway and autophagy process present in *C. elegans* were monitored upon heat stress followed by pathogen exposure. *tol-1* is Toll interleukin receptor coding gene, helps in activating immune cascade via Mitogen Activated Protein kinase pathway (MAPK) (Kamala et al., 2016). *tol-1* has been previously reported to play a crucial role in mounting an appropriate immune response against broad range of pathogens like *S. enterica* (Tenor and Aballay, 2008) and *E. faecalis* (Garsin et al., 2001). A greater emphasis on *tol-1* in *C. elegans* MAPK pathway was given for the defence mechanism against *Proteus* species (Jebamercy et al, 2013). This study also analyses the expression level of *tol-1* during both the pathogenic exposures. As expected, there was consistent up regulation of *tol-1* in all the heat shock un-induced groups, whereas, in heat shock induced groups it was found to be down regulated (Fig 1.4A and B).
Figure 1.4. Relative expression level of genes involved in (A, B) MAPK pathway and IIS pathway (C, D, E, F) heat shock response and (G) autophagy of nematodes infected individually with *P. mirabilis* and *S. aureus* in the presence and absence of heat shock induction at various time periods (12, 24 & 36 h).
Autophagy is considered as one of the major defense mechanisms used to prevent the invading pathogens by guiding the foreign particle for lysosomal degradation (Hoffman et al., 2014). As anticipated, bec-1 gene which codes for autophagy was found to be highly modulated in the early stages of infection (12 h) in both P. mirabilis and S. aureus, when compared to later stages (24 h and 36 h). Notably, bec-1 was prominently up regulated in heat un-induced P. mirabilis exposed samples (Fig 1.4G).

**daf-2 mutant nematodes increases the resistance towards pathogen with heat induction**

daf-2 mutation is well known to have resistance towards pathogens. Moreover, the involvement of daf-2 gene in IIS pathway has long been in aging research (Garsin et al., 2003). The effect of external induction on the longevity of the daf-2 mutant nematodes was investigated in the present study. Surprisingly, heat induction boosted the immune system of these daf-2 mutant nematodes during the exposure of S. aureus with a survival rate of 146 ± 2 h from that of the un-induced nematodes which had a survival rate of 136 ± 2 h (Fig 1.5). An 18 h increase the life span of daf-2 mutant nematodes towards S. aureus infection, which is twice as that of wild type nematodes. However, only 10 h increase in life span was observed in N2. This observation instigated the present study to correlate the repercussion of heat shock on IIS pathway.
Figure 1.5. Survival of *daf-2* mutant induced with heat shock and subsequently infected individually with *S. aureus* (*p*<0.05) and *P. mirabilis* (*p*<0.05). *E. coli* OP50 food source served as control.

**SGK-1 plays the mediator for heat induction dependent longevity**

Immunobloting experiments were carried out to determine the protein expression level of HSF-1, HSP-90 and SGK-1 in N2 and *daf-2* mutant nematodes. HSF-1 generally acts as transcriptional factor that targets HSPs. Therefore, both HSF-1 and HSP-90 were taken for the protein level quantification. In addition to it, SGK-1 which signals HSF-1 by acting equivalent to it was also checked for the protein expression. The blot clearly depicts that SGK-1 is up regulated during heat shock treatment in both N2 and *daf-2* mutant during pathogenic exposure (Fig 1.6A). The quantification graphs also showed up regulation of all three isoforms of SGK-1 in N2 more than *daf-2* mutant nematodes during *S. aureus* infection indicating that SGK-1 mediated activation of transcription factor DAF-16 (Fig 1.6B). The HSF-1 level is up regulated in wild type better than *daf-2* mutant and identical regulation was detected in HSP-90 (Fig 1.6C and 4D). The higher expression level of HSF-1 in the N2 also persuades on the notion that the protein chaperone HSP-90 is evoked by the exertion of HSF-1 nuclear translocation.
Figure 1.6. (A) Western blot depicting the regulation of SGK-1, HSF-1 and HSP90 proteins in wild type and daf-2 mutants infected with S. aureus in the presence and absence of heat shock pre-treatment. β-actin was used as reference control. Relative intensity profile of (B) SGK-1 (including its three isoforms), (C) HSF-1 and (D) HSP-90 with heat shock induction compared to control (without heat shock induction).

Mutation in hsf-1 & hsp-90 severely reduced the life span of the nematode

To check if the mutation in hsf-1 and hsp-90 disturbs the longevity of the nematodes, the survival rate of heat induced hsf-1 and hsp-90 mutant nematodes upon pathogenic
exposure was observed. Interestingly, heat un-induced nematodes survival was higher when compared to heat induced nematodes. The *hsf*-1, OG532 (Fig 1.7A) mutant nematodes induced by external heat stress and with a sequential exposure of *S. aureus* had an early mortality with complete killing at 65 ± 2 h, while *hsp*-90 mutants, PR673 (Fig 1.7B) and JT6130 (Fig 1.7C) was at 60 ± 2 h for the former and 65 ± 2 h for the latter. In stark contrast, the nematodes exposed to *P. mirabilis* and *E. coli* OP50 had similar survival rate in both N2 and the mutant nematodes.

**Figure 1.7.** Survival of (A) *hsp*-90, PR673 (B) *hsp*-90, JT6130 (C) *hsf*-1 mutants induced with heat shock and subsequently infected individually with *S. aureus* and *P. mirabilis*. Nematodes fed on *E. coli* OP50 were considered as control. Significant difference was observed in (B) and (C) that were induced with heat shock and subsequently infected with *S. aureus* (*p*<0.05).
Discussion

Natural environments greatly influence the lifestyle of *C. elegans* and often involve transient exposure to stressful conditions (Gouvea et al., 2015). Seasonal changes in connection with predators, food shortages, and severe weather produce incessant changes in the host system. Nevertheless, the *C. elegans* is equipped with strategies to handle the unpredictable changes. The optimum temperature for the growth of *C. elegans* is 15–25 °C and the temperature range of 30–35 °C is considered as stressors (Cypser and Johnson, 2002). Induction of a set of stress genes (HSPs) involves in protein homeostasis and cellular repair. HSP-90 is bound to HSF-1, which is the prime receiver of heat shock, which when accelerated facilitates maintenance of client proteins important for stress resistance (Kumsta et al., 2013; Chiang et al., 2012). Heat stress was reported to provide favourable effects to *C. elegans* induced at 35 °C for 2 h (Cypser and Johnson, 2002). Besides, persistent exposure to the temperature killed the nematodes (Zhou et al., 2011). On the other hand, IIS pathway was widely known to be involved in aging and its role in pathogenic resistance (Garsin et al., 2003; Kapahi et al., 2010). HSPs are long been proposed as biomarkers of damage, but the connection between activation of HSPs and the IIS is still vague. The goal of the present study is to find the association between IIS pathway and heat shock response. In addition, the hypothesis that external heat shock facilitates pathogenic resistance was also examined.

The longevity of the host upon heat induction and subsequent pathogenic exposure (*S. aureus*, a pathogen proven to be lethal to *C. elegans*) and (*P. mirabilis*, an opportunistic pathogen proven to be non-lethal to *C. elegans*) was determined by survival assay. A significant amount of resistance was observed towards *S. aureus*. However, *P. mirabilis* and the laboratory food source *E. coli* OP50 showed no such increase in life span (Fig 1.1). Sifri et al., 2003 has also reported that a different strain of *S. aureus* makes *C. elegans* mortal at 90 h. The results of Jebamercy et al., 2011 also corresponds to the same as in the present study.
Pathogenicity of *S. aureus* and opportunism of *P. mirabilis* are two major factors to be taken into account for the difference in the survival of nematode during heat induction. The heat induction did not alter the food choice of the nematode (Fig 1.2A and B). The microscopic examination also corresponds to higher infection rate of *S. aureus* in the nematode while the heat shock is un-induced (Fig 1.3). When it was identified that heat induction persuades longevity in N2, the similar hypothesis was checked in *daf*-2 mutant nematodes too. As it was anticipated, *daf*-2 mutants increased its lifespan further with the stimulation of external heat shock (Fig 1.5).

*daf*-2 mutation dramatically affects the IIS pathway by entering the dauer stage and presumably lives for more than two months. Thus, *daf*-2 gene is key factor for several important aspects of aging and longevity. Expression of high level of antioxidant enzymes and HSPs, resistance to oxidative stress are few typical characters of dauer larvae (Gami and Wolkow. 2006). The increase in the survival rate of both the N2 and *daf*-2 mutant nematodes was thought to be directly proportional to the heat shock applied. Since heat shock was given to all the exposures, the heat shock pathway would have activated in all of them. Hence, the enhanced life span observed in *S. aureus* exposed nematodes was probably due to the combined effect of IIS pathway and heat shock pathway and neither of it alone. This initial result, which speculates a positive link between IIS pathway and heat shock pathway, impelled us to discover the molecular mechanism involved in it.

For the molecular level analysis, real time quantification of representative genes involved in both the pathways was performed. Mitogen activated protein kinases (MAPK) acts as the first innate immune response that activates central signaling hubs, executing extracellular processes such as proliferation, differentiation, apoptosis and migration (Andrusiak and Jin, 2016). In order to determine if the heat shock mediated pathogenic
resistance is via MAPK pathway, expression of tol-1, the prime receptor of the pathway was analyzed upon heat shock induction and subsequent pathogenic exposure. The up regulation of tol-1 in the heat un-induced samples suggests the activation of MAPK pathway when there is a pathogenic encounter. tol-1 was already implicated to provide immunity to wide range of pathogens (Pujol et al., 2001; Vigneshkumar et al., 2011; Kamala et al., 2015). Increase in levels of tol-1 was prominent at the initial stage of infection of P. mirabilis and at the later stage of S. aureus. This indicated that the nematode has raised a response earlier consequently suppressing the pathogenesis for the former, while, the host was not able to elicit a response proving its pathogenicity towards the nematode for the latter. Surprisingly, tol-1 was found to be down regulated in heat induced samples (Fig 1.6A and B), providing evidence that heat shock mediated pathogenic resistance is not related to MAPK pathway.

The elimination of MAPK pathway prompted the present study to check on for IIS, which is a classical pathway for aging in C. elegans. Therefore, the expression of daf-2 (receptor of IIS) and age-1 (the adapter protein of IIS), was analyzed to conclude its role in pathogenic resistance and longevity. In the heat induced samples with subsequent S. aureus exposure daf-2 level was down regulated in all the three time points (Fig 1.6A and B). Earlier study has provided evidence that daf-2 RNAi doubled the lifespan of nematodes (Libina et al., 2003). The present study goes in concordance with the study mentioned above for the observed longevity. daf-2 down regulation in S. aureus exposed heat induced samples led to enhanced life span. age-1, the downstream adapter in IIS pathway shared similar grounds as daf-2 and was found to be down regulated in heat induced, S. aureus exposed samples (Fig 1.6A and B). The expression level tol-1, daf-2 and age-1 makes it clear that heat shock mediates pathogenic resistance by associating with IIS pathway.
Analysis of candidate heat shock genes during heat shock induction exhibited elevated levels of \textit{hsp}-1 and \textit{hsp}-6 in the case of \textit{S. aureus} and \textit{P. mirabilis} infection at initial hour (Fig 1.6E and F). This implies the necessity of the chaperonic molecule to ameliorate the damage caused by heat shock. However, the expression of \textit{daf-21} was scarcely affected by heat stress (Fig 1.6E and F). Reports by Gaiser et al, 2012 goes in unison with the the present study, stating that weakened expression level of \textit{daf-21} does not affect the longevity of nematodes rather a combined effect of HSPs provided a proper immune response against the pathogen. Equivalent to the regulation pattern of \textit{daf-16}, \textit{sgk-1} gene expressions in the heat induced samples and with consecutive pathogenic exposures suggested the importance of \textit{sgk-1} for the regulation of the \textit{daf-16} transcription factor. Interrogation of \textit{bec-1} gene in extension of lifespan was also assessed in this study, since autophagy was shown to play a critical role in several nutrient-sensing longevity processes, including insulin-IGF-1 signaling pathways (Edwards et al., 2015) and TOR (Hansen et al., 2008) as well as in the dietary-restriction paradigm. \textit{bec-1} was found to be considerably up regulated in \textit{P. mirabilis} exposed sample than \textit{S. aureus} exposed sample, in the absence of heat induction (Fig 1.6G). A convincing speculation could be that, the significant up regulation of \textit{bec-1} proficiently persuaded autophagy and saved the nematodes from \textit{P. mirabilis} infection. On the other hand, there was very diminutive induction level of \textit{bec-1} observed in \textit{S. aureus} which was not enough to confer such resistance.

Even though the transcriptome level data correlates the heat shock response and pathogen resistance, the protein level confirmation of the hypothesis was further scrutinized. SGK-1 was selected as a representative of IIS pathway, since it has been shown to up regulate during heat induction in the real time analysis. Additionally, HSF-1 was selected with the justification that it plays the core regulator which controls the activation of other
HSPs chaperone (Zhou et al., 2011). Finally, HSP-90 was chosen as a representation for the subset of HSPs and to validate the results of real-time PCR.

Protein quantification of HSF-1 and SGK-1 reflected the real time results wherein they were found to be up regulated in heat shock induced samples of both the N2 and daf-2 mutants (Fig 1.6A, B and C). In stark contrast, HSP-90 was observed to be up regulated at the protein level (Fig 1.6A and D). A speculated reason could be that HSP-90 may be under the influence of certain post-transcriptional regulation. Yet, this study has to be taken for additional validation. With the data obtained it is presumed that SGK-1 acts as the missing piece of puzzle that connects IIS pathway and heat shock response. There have been many reports stating the significance of SGK-1 in modulating longevity and how HSP-90 is required for the phosphorylation and activation of SGK-1 (Mizunuma et al, 2014; Belova et al, 2008). Yet another study by McColl et al, 2010 published that, the global mRNA levels of quite a lot of HSPs are significantly higher in IIS mutants. The present study put forth the idea that the expression of certain HSF-1 and HSP targets during heat induction are at least partially accountable for the lifespan extension against pathogens and observed in IIS pathway. Altogether the observations suggest that up regulation of SGK-1 in the event of heat shock was not mediated via daf-2. There is a greater possibility that HPS-90 which has got up regulated upon heat shock might have activated SGK-1, which further activated its downstream regulators including DAF-16. This proof further strengthens the earlier hypothesis that IIS pathway and heat shock pathway together have played role for the enhanced life span observed upon pathogen encounter. The proposed pathway for the boosted life span witnessed upon pathogenic exposure in heat shock induced nematodes mediated by heat shock and IIS pathway is illustrated in (Fig 1.8).
Lastly, in order to further confirm the obtained results *hsf-1* and *hsp*-90 mutants were checked for its survival during heat shock and consequent pathogenic exposure. The results convincingly proposed that mutation in both *hsf*-1 and *hsp*-90 shortens the lifespan of nematodes (Fig 1.7A, B and C). Morley and Morimoto, 2004 reports was also consistent with the current results wherein, *hsf*-1 mutants have decreased life span. Overall, these results confirm that, in addition to role in longevity, heat shock pathway was also associated to pathogenic resistance and immunity.

![Schematic representation of the plausible role of HSF-1 in inducing the longevity upon heat induction and consequent pathogenic exposure.](image)

**Figure 1.8.** Schematic representation of the plausible role of HSF-1 in inducing the longevity upon heat induction and consequent pathogenic exposure.