Proteomic analysis of *Caenorhabditis elegans* against *Salmonella* Typhi toxic proteins

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Abstract

Background: Bacterial effector molecules are the crucial infectious agents and are sufficient to cause pathogenesis. In the present study, pathogenesis of *Salmonella* enteric serovar Typhi (*S. Typhi*) toxic proteins on the model host *Caenorhabditis elegans* was investigated by exploring the host regulatory proteins during infection through quantitative proteomics approach. In this regard, the host protein extract was analysed using two-dimensional gel electrophoresis (2D-GE) and differentially regulated proteins were identified using MALDI TOF/TOF/MS analysis. **Results:** Out of the 150 regulated proteins identified, 95 proteins were appeared to be downregulated while 55 were upregulated. Interaction network for regulated proteins was predicted using STRING tool. Most of the downregulated proteins were found to be involved in muscle contraction, locomotion, energy hydrolysis, lipid synthesis, serine/threonine kinase activity, oxidoreductase activity and protein unfolding and upregulated proteins were found to be involved in oxidative stress pathways. Hence, cellular stress generated by *S. Typhi* protein extract on the model host was determined using lipid peroxidation, oxidant and antioxidant assays. In addition to that the candidate proteins resulted from the host protein extract analysis were validated by Western blotting and roles of several crucial molecular players were analyzed in vivo using wild type and mutant *C. elegans*. **Conclusions:** To the best of our knowledge, this is the first study to report the protein regulation in host *C. elegans* during *S. Typhi* toxic proteins exposure which highlights the significance of p38 MAPK and JNK immune pathways. **Keywords:** *Caenorhabditis elegans*, Bacterial toxic proteins, 2D-GE, MALDI-TOF-TOF-MS, oxidative stress pathways, Western blotting, immune pathways

1. Background

C. elegans is a well suited model to investigate the cellular impact of bacterial toxic proteins since the model has been well established for its utility in toxicological studies. Its short generation time, large brood size, conventional biology and well defined innate immune system have made it as a suitable model for toxicological studies (Popham *et al.*, 1979; Hodgkin *et al.*, 2000; Leung *et al.*, 2008). Toxicity analysis on *C. elegans* provides the data of whole animal with intact and metabolically active reproductive, digestive, endocrine, sensory and neuromuscular systems (Hunt *et al.*, 2017). Most importantly, *C. elegans* provides a naive and rapid biological system to investigate bacterial toxins and host interaction as it naturally feeds on bacteria (Huffman *et al.*, 2004). Many Gram-negative and Gram-positive bacteria which are pathogenic to humans are also reported to infect *C. elegans* (Aballay *et al.*, 2003). Bacterial pathogens promote host pathogenesis by prominent virulent factors including lipopolysaccharide, flagella, pili, proteases, exotoxin A and exoenzymes (Lyczak *et al.*, 2000). Phenazine (virulence factors) toxicity through oxidative stress was first identified in *C. elegans* (Cezairliyan *et al.*, 2013) which was later reported in *Drosophila*, mice and plants also (Ray *et al.*, 2015). The viable cultures of *S. Typhi*, *P. aeruginosa* and *K. pneumonia* and their isolated lipopolysaccharide (LPS) act as a powerful immune activator and lethal agent on this nematode model (Vigneshkumar *et al.*, 2012; Sivamaruthi *et al.*, 2014; Kamaladevi *et al.*, 2016). It was reported that the lipoteichoic acid of Gram-positive bacteria is equivalently antigenic to LPS which elicit the inflammatory response in the host (JebaMercy *et al.*, 2015).

Our previously reports attest the utility of *C. elegans* as a model organism for the following human pathogens, *Shigella* spp, *Vibrio alginolyticus*, *Proteus* spp. and *S. Typhi* (Kesika *et al.*, 2011; Durai *et al.*, 2011; Kesika and Balamurugan 2012; JebaMercy *et al.*, 2013; Sivamaruthi *et al.*, 2014). These reports uncover the molecular players responsible for host defence upon *K. pneumonia* and *P. aeruginosa* pathogenesis through proteomic approach (Balasubramanian *et al.*, 2016; Kamaladevi and Balamurugan 2017). *Salmonella* spp., are responsible for millions of infections per year ranging from food poisoning to life-threatening systemic typhoid fever (Gal-Mor *et al.*, 2014). *S. Typhi* resistance to antibiotics is an increasing problem, especially in the Indian subcontinent and Southeast Asia (Thaver *et al.* 2008). Typhoid resistance to antibiotic is known as multidrug-resistant (MDR) typhoid ((Zaki and Karande, 2011). For pathogenesis, *Salmonella* spp. uses Type-III Secretion System (T3SS), to deliver bacterial proteins/toxins directly into eukaryotic host cells and amends its cellular functions (Galan *et al.*, 2001). In particular, *S. Typhi* genome encodes ~ 4500 proteins identified by protein analysis (Liu *et al.*, 2015). Toxic proteins are poisonous substance and are capable of causing diseases on contact with or absorption by host body tissues, these proteins interact with biological macromolecules such as enzymes or cellular receptors and disable the host immune system (Smith *et al.*, 1972; Schlesinger D 1975). Hence, in the current study we have investigated the impact of whole protein extract of *S. Typhi* on translational machinery of *C. elegans* by employing proteomic approaches. In this context, *S. Typhi* whole-cell enriched proteins were used to treat *C. elegans* and subsequently the host proteins were isolated and analysed using 2D-GE. To the best of our knowledge, this is the first study to identify differentially regulated candidate proteins in *C. elegans* against bacterial toxins using proteomic approach.

2. Materials And Methods

2.1 Maintenance of nematode, *C. elegans* and bacterial strains

Nematodes used in this study were wild-type N2 Bristol and out crossed mutants, *myo-2* (EG5568), *col-19* (TP12), obtained from the CGC (Caenorhabditis Genetic Centre MN, USA). Synchronized L4 stage worms used for *in vivo* exposure experiments were obtained by bleaching, as described previously Theresa Stiernagle (2006). The L4 stage worms were washed with M9 buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl and 1 mL of 1 M MgSO₄ to 1000 mL H₂O sterilized by autoclaving) and used for all the *in vivo* exposure experiments. All physiological assays including short time exposure were performed using isolated toxin proteins of *S. enteric serovar Typhi* (MTCC 733), which was purchased from Microbial Type Culture Collection and Gene Bank, Chandigarh, India (MTCC). All strains were maintained frozen at -80 °C in the peptone solution (2% peptone, 5% glycerol). The frozen bacteria were usually grown on LB plates with 1.5% agar at 37 °C. A single colony picked from the plate was inoculated into 3 mL LB medium, and then the overnight culture was diluted 1:20 into 300 mL LB broth (with 0.3 M NaCl to increase bacterial invasion).

2.2 Ammonium sulphate (AS) fractionation

The soluble proteins from the *S. Typhi* and *E. coli* OP50 bacteria pellets were obtained by suspending the microbial cell pellets in 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.44 g KH₂PO₄, pH 7.5), disrupted with an ultrasonicator (Sonics & Materials, Danbury, CT, USA) and centrifuged at 17000 × g for 20 min at 4 °C. Before precipitation, the supernatant was filtered by using 0.2 micron filter paper. *S. Typhi* whole-cell extracted proteins were enriched by (NH₄)₂SO₄ precipitation for concentrating the protein samples as per (Wingfield *et al.*, 2001., Park *et al.*, 2008) and also the EnCor Biotechnology Inc (<https://www.encorbio.com/protocols/AM-SO4>) method which conveniently determined the amount of solid ammonium sulphate required to reach a given saturation. The precipitated protein fractions were desalted by Zeba spin centrifuge column (ThermoFisher, product code – 89889) based protocol. The toxicity assays including quantitative growth, embryonic development, oxidant and antioxidant assays and lethal dose assays were performed in liquid media. The Ammonium nitrogen in solution was estimated by phenate method as per Park *et al.*; 2009 with the assay medium contains 0.025 µg/mL of Ammonium nitrogen.

2.3 Physiological studies during exposure with *S. Typhi* toxin proteins in *C. elegans*

2.3.1 Nematode liquid killing assay

C. elegans killing assay was performed to determine the impact of extracted proteins of *S. Typhi* on nematode. Approximately, 20 L4 stage age-synchronized *C. elegans* were transferred to a 24-well plate containing *E. coli* OP50 bacteria (positive control), *E. coli* OP50 extracted protein plus *E. coli* OP50 bacteria (negative control) and *S. Typhi* extracted protein plus *E. coli* OP50 bacteria (treated) respectively. The plates were incubated at 20 °C and scored for viability of *C. elegans* every 6 hrs. Worms were considered dead upon failure to respond upon gentle touch using a worm picker containing platinum wire on the solid NGM plates. All the experiments were carried out in triplicates. Kaplan-Meier survival analysis was used to compare the mean lifespan of control and treated nematodes. The experiment was performed in biological triplicate, and the error bars represent the mean ± SD (**p* < 0.05).

2.3.2 *C. elegans* protein sample preparations

After 24 hrs of exposure *C. elegans* positive control and treated, worms were washed thoroughly with M9 buffer to take away the surface bound proteins and bacteria. The washed worms were immersed in 50 mM Tris-HCl buffer (pH 8.5 along with protease inhibitor cocktail) then sonicated on ice for 3 min at 10 second pulse interval, debris were removed by centrifugation at 7000 × g for 5 min and subsequently, the resulting supernatant was collected and purified using a 2D cleanup kit (GE Healthcare) as per manufacturer's protocol. The Protein concentration was determined using Bradford reagent (Sigma Aldrich) (Bradford *et al.*, 1976) and the protein concentration was maintained at 1 mg per sample. The samples were dissolved in (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Dithiothreitol (DTT) and carrier ampholytes 2% v/v added (per manufactures protocol) and subjected to 2D-GE.

2.3.3 Iso-electric focusing (IEF) and 2D-GE

During IEF (first dimension), proteins were separated based on their isoelectric point on 18-cm immobilized pH gradient (IPG) gel strips of pH 3–10 (GE Healthcare). Subsequent to overnight rehydration, IPG strips were subjected to IEF at 20°C under mineral oil with the following conditions: 3 hrs at 100V; 1 h at 500V; 1 hrs at 1000V; 2 hrs at 1000-5000V (gradient); 1 h at 5000V; 3 hrs at 5000-10000V (gradient) and final focussing was done for 2 hrs at 10000V. The current was set to 75 µA per IPG strip. Prior to SDS-PAGE, IPG strips were immersed twice for 15 min in equilibration buffer I [6 M urea, 30% (w/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS) and 1% (w/v) DTT in 50 mM Tris-HCl buffer, pH 8.8] followed by equilibration buffer II [6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 2.5% (w/v) iodacetamide (IAA) in 50 mM Tris-HCl buffer, pH 8.8]. After equilibration, proteins were separated based on their molecular weight using 12.5% SDS-PAGE (second dimension). Electrophoresis was performed at 100V (200 mA) for 1 h and 150V (300 mA) for 7–8 hrs in Ettan DALT six apparatus (GE Healthcare). After electrophoresis, gels were kept in fixative solution (containing 40% methanol, 10% glacial acetic acid and 50% Milli Q H₂O) for overnight and washed thrice with Milli-Q H₂O for 20 min each. Protein spots were stained by colloidal coomassie brilliant blue

(CBB) G-250 staining solution (containing 10% ortho phosphoric acid, 10% ammonium sulphate, 20% methanol and 0.12% CBB) for 12 hrs on rotary agitator. Subsequently, the gels were destained with Milli-Q H₂O for 4 hrs to reduce the background noise.

2.3.4 Trypsin digestion of differentially regulated protein spots and mass spectrometric analysis

Destained gels were scanned with a densitometry image scanner (Image Scanner III, GE Healthcare) at 300 dpi resolution and captured using Lab Scan 6.0 software. The raw images were analysed using Image Master 2D Platinum 7 (GE Health care). Based on the densitometry analysis of gel images which detected about total 898 protein spots amongst, 477 spots were satisfied the arbitrary parameters in control and treated gels. Interested spots with more than 1.5 fold changes in the intensity were excised manually. Subsequently, prior to in-gel trypsin digestion, the excised proteins were completely destained by washing with destaining solution (containing 50% acetonitrile and 25 mM ammonium bicarbonate) and dehydrated in 100% acetonitrile (ACN) for 10 min, then dried under vacuum for 30 min. After reduction and alkylation as per the standard protocol, in-gel trypsinisation was performed with 5 µL of trypsin buffer (10 mM NH₄HCO₃ in 10% ACN) containing 80 ng of trypsin (Sigma Aldrich) and incubated at 37°C for 16 hrs. After incubation, peptides were extracted by 0.1% trifluoroacetic acid (TFA) in 60% ACN by bath sonication (10 min) subsequently dehydrated by 100% ACN and extracted peptides were dried under vacuum for 90 min at 45°C. For MALDI-TOF/TOF/MS analysis dried peptides were dissolved in peptide resuspension solution (0.1% TFA in 5% ACN) and desalted/concentrated using C18 zip tips (Merck Millipore) as per the manufacturer's protocol. An equivalent volume (1 µL) of C18 zip tip purified peptides were mixed with a matrix solution (containing 10 mg/mL of α-cyano-4-hydroxy cinnamic acid matrix in 1 mL of 60% methanol-0.1% formic acid) and spotted on an Anchorchip target plate. Calibration was performed with TOF- Mix™ (LaserBio Labs, France) as an external standard. The peptide mass was analysed with a MALDI-TOF mass spectrometer (AXIMA Performance, SHIMADZU BIOTECH) in positive reflector ion mode and analysed by Shimadzu launch pad- MADLI MS software. Mono isotopic peak list (m/z range of 700–4000 kDa with S/N ratio over 10) was generated by MALDI MS software. Peptide mass fingerprints (PMFs) were analysed using online MASCOT server. When searching MASCOT, Swissprot database was mined against PMFs and MALDI-TOF/TOF/MS as instrument, *C. elegans* as the organism source, selected variable modification were carbamidomethylation of cysteine, oxidation, N-terminal acetylation and phosphorylation (S, T, Y) for methionine, whereas a maximum of one missed cleavage sites was allowed and mass tolerance of 100 ppm per peptide was set as fixed modifications (Ananthi et al., 2011; Sethupathy et al., 2016).

2.3.5 Bioinformatics analysis

Regulatory proteins identified from high throughput analysis were further subjected to bioinformatics analysis such as gene ontology (GO) classification in UniProt KB tool and interaction among them was assessed using STRING with medium confidence score 0.400. The gene enrichment score and functional annotation was generated using DAVID tool (Kamaladevi et al., 2017).

2.3.6 *In vitro* embryo development toxicity assay

C. elegans embryos were obtained from gravid adult worms per protocol (Bianchi et al., 2006). Briefly, the worms from NGM agar plates were washed by Milli-Q water pellet down by centrifugation at 170 × g for 3 min. The pellets were lysed using the mixture of bleach and NaOH (Fresh Chlorox 5 mL, 10 N NaOH 1.25 mL and sterile H₂O 18.75 mL) incubated for 5 min by giving gentle vortex several times and centrifuged at 170 × g for 3 min. The pellets and eggs were washed with egg buffer (118 mM NaCl, 48 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, and 25 mM Hepes pH 7.3) and centrifuged at 170 × g for 3 min. The pelleted eggs were resuspended and lysed in 2 mL of sterile egg buffer and 2 mL of a sterile 60% sucrose (in egg buffer) by vortexing. The suspension was centrifuged at 170 × g for 5–6 min. The eggs were collected from the top of the solution; approximately isolated 1000 oocytes/eggs were exposed in a 24-well microtitre plate containing *E. coli* OP50 bacteria (control) or *S. Typhi* extracted proteins (treated).

2.3.7 Detection of oxidants and antioxidants

The L4 stage *C. elegans* exposed to positive control, negative control and *S. Typhi* extracted protein (treated) were washed several times thoroughly. Subsequently, the bacteria free worms were homogenized and protein concentration was maintained at 100 µg. Reactive oxygen species (ROS) was measured per Scherz-Shouval et al. (2007) to study the level of ROS in host during *S. Typhi* extracted protein exposure. The H₂O₂ level in cell lysate supernatant was measured as described earlier Wolff et al. (1994). SOD activity of *C. elegans* cell lysate supernatant (100 µg of protein) was measured as per Paoletti et al., 1990. Catalase activity of *C. elegans* was measured as described earlier Aebi et al., 1984. *C. elegans* carbonyl content was measured as per Levine et al., 1990. Lipid peroxidation was determined as described earlier Ohkawa et al., 1979. Each experiment was performed in biological triplicates and the error bars represent the mean ± SD (**p* < 0.05).

2.3.8 ATP assay

Total intracellular ATP of *C. elegans* control and treated samples was measured as described earlier Chen *et al.*, 1994. The protein concentration of the supernatant was determined and protein concentration was maintained at 500 µg (Bradford's method) for all the three time points in triplicate. The ATP concentration in each sample was calculated according to the given formula:

$$\text{ATP (M)} = \frac{\text{Standard (RLU)} - \text{Balance reagent (RLU)}}{\text{Unknown (RLU)}} \times \text{Amount of ATP standard added (M)}$$

2.3.9 Behavioural assay

This assay was performed to determine the impact of food source (*E. coli* OP50) on *S. Typhi* protein treated *C. elegans* life span, locomotion and reproduction. The experiment was analysed *in vivo* using *C. elegans*. The age synchronized L4 stage N2 worms treated with toxin proteins (concentration 500 µg/mL, 1 mg/mL and 1.5 mg/mL) respectively, were incubated at 20 °C for 12 hrs. After incubation the treated worms were washed with M9 buffer several times to remove traces of protein from the body and transferred to NGM agar plates seeded with food source *E. coli* OP50. The plates were incubated at 20 °C and *C. elegans* life span, locomotion and reproduction were monitored during the assays. The plates were scored for viability of *C. elegans* after every 2 hrs. Worms treated with *E. coli* OP50 were considered as control. Each experiment was performed in biological triplicates and the error bars represent the mean ± SD (**p* < 0.05).

2.3.10 Western Blotting

The protein content of whole cell extracts (at different time points of exposure) were prepared using lysis solution (containing 7 M urea, 2 M thiourea, 4% w/v CHAPS and 30 mM Tris-HCl, pH 8.5 along with protease inhibitor cocktail). Protein concentration was determined by Bradford assay. 100 µg of protein for each sample, was boiled in 5X Laemmli buffer (0.3125 M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.005% bromophenol blue, 25% beta-mercaptoethanol) for 5 min followed by short spin at 1100 x g. The protein samples were subjected to SDS-PAGE followed by transfer on nitrocellulose membranes and subsequently, treated to the specific antibodies as described earlier (Durai *et al.*, 2014). The antibodies JNK-1[sc-571], p38[sc-17852], HSP-1[sc-9144], HSP-90 [sc-1055], SGK-1 [sc-33774] used in this study were purchased from Santa Cruz Biotechnology, and beta-actin purified mouse immunoglobulin [A1978] was purchased from (Sigma-Aldrich) working concentration was kept at 1:1000–1:2000.

2.3.11 Statistical analysis

All experiments were performed independently in triplicate. The statistical significance of data was analysed by one-way ANOVA and Duncan's test (SPSS Chicago, IL, USA) at a significance level of *p* < 0.05.

3. Results

3.1 Killing assay

The *S. Typhi* and *E. coli* OP50 extracted proteins were precipitated using (NH₄)₂SO₄ and confirmed by resolving in SDS-PAGE. The protein bands with varying intensity and pattern were seen in precipitated fractions (**Supplemental Fig. 1**). To determine the effect and importance of bacterial proteins on the host-pathogen interaction, killing ability of *S. Typhi* and *E. coli* OP50 precipitated protein was assessed by performing killing assay. The result indicated that *S. Typhi* extracted proteins required 36 ± 5 hrs (*p* < 0.05) for the complete killing of *C. elegans* (Fig. 1A) with the LT₅₀, (time for half to die) of 20 ± 2 hrs. Ammonium sulphate precipitated protein fractions (50% – 80%) killed the *C. elegans* with mean life span 36 ± 5 hrs, whereas *E. coli* OP50 extracted protein fractions (50% – 70%) at same concentration (1.5 mg/mL) have not killed the worms. To confirm whether any other bacterial component is responsible for the *C. elegans* mortality, *S. Typhi* extracted protein fractions was digested with proteinase-K (broad-spectrum of serine proteases which are able to digest the protein extract) overnight at 37 °C and tested for its pathogenicity. The result clearly denoted that there was no significant (*p* < 0.05) difference between mean life span in overnight digested protein fractions and control worms fed with *E. coli* OP50 which suggested that only the protein fractions have modulated the *C. elegans* lifespan and morphology (Fig. 1B). The lethal *S. Typhi* toxin protein fractions (50% – 80%) at 1.5 mg/mL, 1 mg/mL and 500 µg/mL concentration significantly (*p* < 0.05) killed *C. elegans* at 36, 65, and 90 hrs respectively, whereas *E. coli* OP50 and its extracted proteins fraction has not bring any significant physiological changes in the nematode (Fig. 1C). To confirm the (NH₄)₂SO₄ effect on nematode, the L4 stage N2 worms were grown in various concentrations of (NH₄)₂SO₄ medium and monitored for their survival rate. It was found that (NH₄)₂SO₄ medium exceeding the concentration of 500 mM is lethal to *C. elegans* (Fig. 1D). Each experiment was performed in biological triplicates and the error bars represent the mean ± SD (**p* < 0.05).

3.2 2D-GE based proteomic analyses of *C. elegans* upon exposure to toxins

The results of the killing assay and protein pattern of *C. elegans* on SDS-PAGE (**Supplemental Fig. 2**) have lead us to investigate the primary molecular mechanism of *C. elegans* mortality through 2D-GE. Worms exposed to *S. Typhi* toxins for 24 hrs were taken for the analysis. A 2D-GE was deployed to decipher protein regulation in control and treated samples respectively (Fig. 2A and B). The 2D-GE triplicate gels are shown in Supplemental Fig. 3. The protein spots present in the control and treated 2D-GE gels were matched and compared using Image Master Platinum 7 software (GE Healthcare). Based on the densitometry analysis 477 detected protein spots were found to satisfy the arbitrary parameters. The relative expression ratio of downregulated and upregulated protein spots in control and treated sample was fixed at ≥ -1.5 and ≥ 1.5 respectively of all the biological replicates ($p < 0.05$). Among 477 matched spots, 95 and 55 spots were found to be downregulated and upregulated, respectively. Selected differentially expressed protein spots were excised from preparative gel and analysed by MALDI-TOF/TOF/MS, and proteins were identified by Mascot tool. The list of identified differentially regulated proteins with their Mascot score, percentage of sequence matched and fold change is provided in Tables 1 and 2. A GO classification of regulated proteins was performed using the UniProtKB tool to categorize regulated proteins into, biological processes, molecular functions and cellular components. The functional annotations of largest set of regulated proteins are presented in (Fig. 3). Most of the identified regulated proteins have shown to play important roles in embryology, cytoskeleton, reproduction, metabolism development, ubiquitination, and oxidative stress. All these biological processes are directly reliable response to changes made by stress conditions. The interaction among regulated protein players of *C. elegans* was performed using the STRING tool. The interaction analysis was performed to decipher high degree of connectivity and their role in important biological pathways. The interaction map displayed the relation between the identified regulated proteins of *C. elegans* (Fig. 4). The downregulated protein DAF-21 showed interaction with MYO-1/2, UNC-54, LET-754, H28O16.1, STI-1, KGB-1, TRX-1 and HSP-16.2 proteins (Fig. 4A). These proteins play an important role in muscle contraction (*myo-1/2*), locomotion (*unc-54*), energy hydrolysis (*let-754*), ATP synthase complex (H28O16.1), stress response (*sti-1*), serine/threonine kinase activity (*kgb-1*), oxidoreductase activity (*trx-1*) and protein unfolding (*hsp-16.2*). STRING analysis of upregulated proteins displayed the interaction between SOD-1, CTL-1, PXN-1, GCK-1 and SEK-1 which is ubiquitously related to oxidative stress (Fig. 4B). Functional annotation and gene enrichment of all regulated proteins [N = 150] were performed using DAVID tool. Functional annotation reflects the character of one protein in diverse biological processes. The biological functions whose highest numbers of proteins that are differentially regulated in response to *S. Typhi* extracted proteins exposure were embryonic development [N = 56], post embryonic development [N = 38] as provided in (Fig. 5A). Biological process that exhibits highest gene enrichment score were cytoskeleton, metal binding, cell adhesion, protease and redox processes as provided in (Fig. 5B).

Table 1
List of downregulated proteins present in *C. elegans* (control sample) identified using MALDI-TOF/TOF/MS.

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
1	Adenylate kinase	let-754	1.5	1	28.0	8.9	10	33	40	0.059
2	BRO1 domain containing protein BROX homolog	B0507.2	1.5	1	48.7	6.0	8	27	20	0.049
3	Actin depolymerizing factor 1	unc-60	1.9	1	23.7	5.5	10	58	71	0.033
4	GLH binding kinase 1	kgb-1	1.7	3	45.2	6.2	27	81	63	0.057
5	NAD dependent protein deacylase sir2.2	sir- 2.2	4.7	2	32.7	7.5	24	109	78	0.048
6	1,2 dihydroxy3-keto-5 methylthiopentene dioxygenase	T01D1.4	1.5	3	18.6	5.5	17	59	69	0.017
7	Obg-like ATPase-1	ola-1	1.8	2	44.6	6.4	21	78	60	0.023
8	Myosin-2	myo-2	1.8	1	22.3	5.9	103	24	42	0.023
9	26S proteasome non ATPase regulatory subunit 3	rpn-3	3.2	1	57.8	7.1	37	45	76	0.005
10	CRALTRIO domain containing protein F28H7.8	F28H7.8	1.6	2	48.0	6.5	19	89	46	0.029
11	Heat shock protein 90	daf-21	2.1	2	90.6	4.9	31	104	44	0.016
12	Spindle assembly abnormal protein 5	sas-5	1.7	2	46.4	8.1	24	75	52	0.044
13	Alpha catenin -like protein hmp-1	hmp-1	2.3	2	104.7	5.6	16	31	19	0.023
14	Polypeptide N-acetyl galactosaminyl transferase-5	gly-5	2.0	3	72.2	6.4	42	134	60	0.027
15	Nuclear hormone receptor family member nhr-106	nhr-106	1.9	2	44.6	8.9	27	80	65	0.041
16	Kelch repeat and BTB domain -contaning protein	F47D12.7	3.0	1	29.9	6.6	25	35	46	0.012
17	Heat shock protein Hsp16.2	hsp-16.2	2.8	1	16.2	5.7	30	86	79	0.057
18	Ectopic P granules protein 2	epg-2	2.1		80.4	4.7	30	43	40	0.057
19	Degenerin like protein asic-2	asic-2	1.6	3	60.4	4.6	10	55	32	0.084
20	PDZ domain containing protein	C52A11.3	2.5	1	14.5	10.2	7	44	57	0.038
21	Profilin-1	pfn-1	4.5	3	14.3	5.1	14	101	87	0.012
22	Triosephosphate isomerase	tpi-1	1.7	1	26.6	6.2	15	35	56	0.025
23	Aurora/IPL1related protein kinase 2	air-2	1.7	1	34.9	8.9	10	36	30	0.054
24	UMP-CMP kinase 2	F40F8.1	1.6	1	21.5	5.8	7	41	49	0.052
25	Peptidyl-prolyl cistrans isomerase 10	cyn-10	1.7	2	18.1	5.2	15	86	100	0.012
26	Cullin-2	cul-2	1.5	1	98.5	8.3	51	137	52	0.012
27	40S ribosomal protein S4	rps-4	4.6	2	29.1	10.4	21	65	51	0.262

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
28	Proteasome subunit alpha type-7	pas-4	1.8	2	46.4	5.9	18	32	77	0.207
29	Mitochondrial import inner membrane translocase subunit TIM1	dnj-21	5.3	4	9.9	11.7	10	79	82	0.011
30	Lipoamide acyltransferase component of branched chain alpha-keto acid-dehydrogenase complex	dbt-1	2.3	3	49.9	8.6	22	88	43	0.037
31	Glutamate-cysteine ligase	gcs-1	2.7	1	75.1	6.2	51	111	54	0.010
32	Probable histone deacetylase complex subunit SAP18	C16C10.4	1.8	2	19.1	9.2	15	75	76	0.036
33	Stress induced phosphor protein1	sti-1	2.5	1	37.8	6.5	34	33	34	0.041
34	Probable 26S protease regulatory subunit	rpt-4	2.7	2	119.1	8.7	27	195	64	0.034
35	Dipeptidyl peptidase family member 1	dpf-1	2.9	4	91.8	5.7	32	140	51	0.054
36	FACT complex subunit SSRP1A	hmg-4	2.2	2	78.9	5.1	38	93	50	0.051
37	Phosphatidyl ethanolamine binding protein homolog	F40A3.3	1.6	2	24.1	8.6	18	83	67	0.003
38	Eukaryotic translation initiation factor 3 subunit A	egl-45	3.3	2	124.6	9.0	37	105	35	0.036
39	Phosphatidyl choline: ceramide choline phosphotransferase 1	sms-1	2.1	1	53.1	6.0	18	68	47	0.039
40	Peptide-N(4)-(N-acetyl-beta-glucosaminyl)asparagine amidase	png-1	3.6	2	70.1	6.7	42	159	42	0.044
41	Tubulin specific chaperone B	tbcb-1	3.4	2	25.5	4.7	26	120	83	0.055
42	Leishmanolysin-like peptidase	Y43F4A.1	1.5	1	77.7	6.0	24	80	41	0.066
43	C-type lectin domain containing protein 161	clcc-161	1.5	2	96.3	5.5	50	199	48	0.049
44	Uncharacterized protein F52C9.3	F52C9.3	2.2	1	50.3	7.8	13	23	31	0.055
45	Putative 6-pyruvoyl tetrahydrobiopterin synthase	ptps-1	4.0	2	16.1	7.7	16	81	73	0.052
46	Putative deoxyribose phosphate aldolase	F09E5.3	1.7	2	33.4	7.0	27	128	76	0.048
47	G- patch domain and KOW motifs- containing protein homolog 1 double check	gkow-1	2.1	1	51.8	5.6	28	68	52	0.036
48	Cell death abnormality protein- 2	ced-2	2.1	2	30.9	5.8	14	88	65	0.050
49	Kinetochore null protein 1	kn1-1	2.0	1	113.4	4.9	28	47	34	0.015
50	Ribonuclease H2 subunit A	rn1-2	1.8	1	65.6	6.4	8	27	25	0.017

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
51	Biogenesis of lysosomerelated organelles complex 1 subunit 6	glo-2	1.6	4	12.0	4.9	7	64	78	0.014
52	MICOS complex subunit MIC19	cope-1	1.5	2	19.4	5.2	15	96	82	0.054
53	Intraflagellar transport protein 56	dyf-13	2.1	1	63.7	7.0	9	108	48	0.038
54	Thioredoxin-1	trx-1	2.7	1	13.6	5.5	5	36	62	0.018
55	ATP synthase subunit alpha	H28O16.1	2.5	1	57.8	8.9	11	40	26	0.035
56	RNA cytidine acetyltransferase	nath-10	5.6	1	117.7	8.4	21	34	30	0.013
57	Protein yippee-like B0546.4	B0546.4	3.8	1	18.3	8.6	19	83	65	0.019
58	Protein pat-12	pat-12	2.3	1	76.6	9.4	43	102	48	0.039
59	Ankyrin repeat and LEM domain containing protein 1	lem-3	1.7	1	78.5	10.2	57	190	60	0.028
60	Zyxin	zyx-1	2.7	1	37.3	8.6	28	111	57	0.059
61	Cytochrome bc-1 complex subunit 1	ucr-1	1.7	2	51.7	6.0	47	178	76	0.441
62	60 kDa SS-A/Ro ribonucleoprotein homolog	rop-1	4.0	1	72.8	6.7	10	30	25	0.095
63	Protein gfi-3	gfi-3	4.5	3	92.7	8.2	27	66	33	0.054
64	Uncharacterized peptidase C1like protein F26E4.3	F26E4.3	1.2	1	52.5	7.2	16	29	29	0.070
65	LIM and SH3 domin protein	F42H10.3	2.6	1	37.5	8.2	8	80	41	0.059
66	Protein timeless homolog	tim-1	2.1	2	157.4	5.3	52	71	37	0.013
67	ATPase inhibitor mai-1	mai-1	2.0	2	95.6	6.4	12	68	81	0.015
68	Probable peroxiredoxin prdx-3	prdx-3	2.0	1	25.2	6.9	8	32	50	0.012
69	Dual specificity mitogen activated protein kinase	mek-1	3.5	1	40.1	6.0	7	28	29	0.010
70	Probable ornithine amino transferase	oatr-1	1.5	1	46.9	8.7	41	130	62	0.014
71	FMRF amide like neuropeptides 1	flp-1	1.2	1	17.7	9.8	9	48	36	0.075
72	Myosin-4	unc-54	2.6	3	22.5	5.5	90	93	34	0.018
73	UPF0160 protein C27H6.8	C27H6.8	6.2	3	38.6	5.4	24	104	46	0.015
74	V-type proton ATPase catalytic subunit	vha-13	1.5	3	66.8	5.0	30	99	46	0.273
75	Putative zinc finger protein R05D3.3	R05D3.3	2.4	1	46.9	6.7	6	33	14	0.041

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
76	Neprilysin-2	nep-21	2.7	1	88.3	7.0	10	29	10	0.029
77	Transcription initiation factor IIB	ttb-1	1.8	2	33.5	8.3	10	34	35	0.042
78	Uncharacterized protein EEED8.2	EEED8.2	12.4	4	35.4	5.5	17	78	56	0.022
79	Alkyl-di-hydroxy-acetone-phosphate synthase	ads-1	1.7	2	67.2	6.3	16	30	40	0.306
80	Exonuclease mut-7	mut-7	1.8	2	106.1	6.5	20	42	79	0.045
81	ATP dependent (S)NAD (P)H-hydrate dehydratase	unc-98	2.7	2	34.1	6.8	29	86	69	0.029
82	D-glucuronyl C5-epimerase	hse-5	1.6	2	70.4	6.1	27	81	54	0.038
83	Lamin-1	lmn-1	5.0	2	64.1	5.4	37	93	53	0.160
84	Protein FMC1 homolog	C29E4.12	3.4	1	12.1	10.0	12	85	73	0.027
85	UPF0376 protein F10G2.1	F10G2.1	1.9	2	23.6	4.9	20	107	78	0.069
86	Nucleoporin SHE 1	npp-18	1.5	1	42.2	8.0	22	90	51	0.049
87	Probable elongation factor 1beta/1delta2	eef-1B.2	2.4	3	28.2	4.9	22	106	79	0.012
88	Kelch-like protein 8	kel-8	1.3	2	77.8	5.6	39	91	43	0.056
89	Trehalose phosphatase	gob-1	4.7	2	52.5	5.1	26	77	50	0.299
90	Mitotic checkpoint protein bub-3	bub-3	1.5	1	38.3	7.7	10	34	23	0.092
91	Tryptophan 2,3dioxygenase	tdo-2	2.3	2	46.8	6.2	29	111	56	0.069
92	Uncharacterized protein E02H1.5	E02H1.5	2.0	1	29.2	6.0	13	29	62	0.021
93	Probable enoyl-CoA hydratase	ech-6	1.9	1	19.3	9.4	8	31	52	0.409
94	Ras-related protein Rab-10	Rab-10	4.5	1	22.9	6.9	14	32	77	0.026
95	Myosin-1	myo-1	5.2	1	22.4	5.7	30	29	19	0.032

Table 2
List of upregulated proteins present in *C. elegans* (treated sample) identified using MALDI-TOF/TOF/MS

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
1	Exocyst complex component 2	sec-5	1.6	1	10.1	5.7	11	33	21	0.040
2	Beta1,3galactosyltransferase bre-5	bre-5	6.0	1	37.7	9.1	10	27	40	0.011
3	Acyl-CoA -dehydrogenase family member 11	acdh-11	3.0	1	68.6	8.4	32	28	41	0.058
4	Uncharacterized monothiol glutaredoxin F10.D7.3	F10.D7.3	1.8	1	16.6	7.82	18	29	81	0.036
5	Probable splicing factor arginine/serine-rich 3	rsp-3	1.6	1	28.7	10.2	27	30	70	0.055
6	Receptor-type guanylate cyclase gcy-8	gcy-8	2.1	1	12.9	6.2	40	25	36	0.017
7	26S protease regulatory subunit 7	rpt-1	1.5	1	49.03	6.0	17	50	31	0.013
8	Rab-3 GTPase-activating protein regulatory	rbg-2	2.7	1	151.1	5.3	83	178	63	0.004
9	Putative uncharacterized protein T27A8.3	T27A8.3	2.0	1	45.2	9.3	10	47	61	0.043
10	Putative neutral sphingo myelinase	T27F6.6	1.6	2	50.2	6.3	15	55	48	0.037
11	Superoxide dismutase [CuZn]	sod-1	2.4	2	18.8	6.1	13	28	53	0.056
12	Dual specificity mitogen activated protein kinase	sek-1	1.7	1	39.1	8.2	21	35	47	0.039
13	Sphingosine-1-phosphate lyase	spl-1	1.9	1	61.6	7.0	10	25	22	0.018
14	Putative carbonic anhydrase-3	cah-3	3.7	1	28.3	5.7	5	26	25	0.051
15	Probable ornithine aminotransferase	smrc-1	2.1	1	46.9	8.7	41	134	58	0.024
16	Ribosomal RNA processing Protein 1	C47E12.7	1.6	1	46.1	9.3	13	27	35	0.056
17	Uncharacterized protein R166.3	R166.3	2.0	1	23.1	8.9	20	76	66	0.039
18	Probable glutathione transferase omega-2	gsto-2	2.4	1	28.8	6.5	15	31	44	0.020
19	mRNA capping enzyme	cel-1	1.9	4	72.6	8.1	40	111	50	0.010
20	Vinculin	deb-1	2.3	1	11.2	6.2	69	34	60	0.041
21	Intraflagellar transport associated protein-1	ifta-2	1.9	2	29.0	4.7	6	27	38	0.013
22	Xaa -Pro aminopeptidase app-1	app-1	3.1	1	69.5	6.1	28	27	47	0.016
23	Uncharacterized protein ZK1073.1	ZK1073.1	1.8	2	35.9	6.8	6	46	21	0.050

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
24	Nuclear hormone receptor family member nhr-153	nhr-153	2.3	1	48.4	9.4	31	30	69	0.021
25	Esterase C25G4.2	C25G4.2	1.6	1	24.8	6.5	21	94	74	0.045
26	GDP fucose protein O-fucosyl transferase-2	pad-2	1.6	2	50.4	6.0	22	59	39	0.042
27	Protein lin-28	lin-28	1.6	2	25.8	6.5	23	97	68	0.037
28	ADP-ribosylation Factor like protein 3	arl-3	1.6	3	20.8	7.7	17	101	83	0.038
29	Uncharacterized protein C05D10.4	C05D10.4	4.6	1	67.8	9.4	41	30	54	0.0461
30	Eukaryotic translation initiation factor	EIF-3.K	1.8	3	27.2	6.4	22	103	69	0.0234
31	UPF0046 protein C25E10.12	C25E10.12	1.5	2	32.2	6.5	18**	70	69	0.019
32	Beta1,4-N -acetyl galactosaminyl transferase bre-4	bre-4	2.5	2	44.3	8.6	13	50	31	0.025
33	Peroxidase skpo-1	skpo-1	4.0	2	74.5	8.7	38	171	65	0.095
34	Catalase-1	ctl-1	2.0	4	57.6	6.4	38	148	63	0.0156
35	Serine/threonineprotein phosphatase Pgam-5	pgam-5	2.5	2	16.7	6.5	12	43	87	0.062
36	UPF0046 protein K07C11.7	K07C11.7	4.6	2	33.1	5.6	13	32	51	0.026
37	Protein phosphatase ppm-1	ppm-1	2.4	2	52.3	5.0	35	102	66	0.044
38	ATPdependent (S)-NAD(P)H-hydrate dehydratase	R107.2	1.8	2	34.1	6.8	24	91	67	0.047
39	DEAD box ATP dependent RNA helicase rde-12	rde-12	1.9	2	104.2	6.7	16	30	24	0.042
40	Putative esterase F42H10.6	F42H10.6	5.5	2	18.4	7.0	17	101	79	0.011
41	40S ribosomal protein S2	rps-2	1.8	1	29.2	10.1	18	32	58	0.023
42	Vesicle-fusing ATPase	nsf-1	2.4	1	91.7	7.2	44	35	56	0.031
43	MICOS complex subunit MIC19	chch-3	1.9	2	19.4	5.4	14	96	76	0.019
44	Germinal center kinase - 1	gck-1	2.9	1	71.4	6.7	35	34	56	0.022
45	Sorting nexin17 homolog	snx-17	1.6	2	63.3	6.8	22*	75	35	0.033
46	Nuclear hormone receptor family member nhr-169	nhr-169	1.0	1	41.9	9.0	25	33	55	0.081
47	Origin recognition complex subunit 2	orc-2	12.3	1	49.4	6.0	23	34	46	0.024
48	Pumilio domain containing protein 12	puf-12	1.6	1	87.4	9.3	36	30	33	0.067
49	MICOS complex subunit MIC60-1	immt-1	4.6	2	76.0	6.6	49	105	46	0.003
50	Partitioning defective protein 3	par-3	3.7	1	14.9	9.1	51	38	46	0.011

Serial No	Protein Name	Gene	Fold Change	No. of Hits	MW (kDa)	PI Value	No. of peptides matched	Mascot Score	Sq. Coverage %	ANOVA
51	Probable actin related protein 2/3 complex subunit 2	arx-4	1.6	2	34.5	7.7	16	66	47	0.015
52	Ubiquitin carboxyl terminal hydrolase 14	usp-14	2.3	1	56.2	5.3	17	140	66	0.069
53	PCI domain containing protein 2 homolog	C27F2.10	1.6	3	47.6	6.1	14	62	57	0.057
54	32 kDa betagalactoside binding lectin lec-3	lec-3	2.1	1	33.6	7.1	10	47	47	0.024
55	Peroxidasin homolog	pxn-1	2.0	1	148.4	8.2	19	28	19	0.056

3.3 *C. elegans* protein classes regulated by *S. Typhi* extracted proteins

3.3.1 Regulation of reproduction and embryo development proteins

The GO analysis showed 56 regulated proteins, which appears to play important role in embryogenesis and reproduction among which the proteins identified responsible for the embryonic development include LIN-28, UNC-60, SPL-1, ZYX, MUT, HMP, Y43F4A.1, STI-1, NPP-1, SAS-5, UNC-98, ZYG-1, PIG-1 CED-2 and TDO-2. Light microscopic images [Nikon Eclipse TI-s, Japan], of treated *C. elegans* showed reproductive failure and produced degenerated embryos compared with that of worms fed with *E. coli* OP50 (control) (Fig. 5C). *C. elegans* embryo cells use to terminally differentiate within 12 hrs of incubation at 20 °C to enter life cycle events (L1 larvae stage). The effect of bacterial toxin protein on nematode embryogenesis was further confirmed by treating the isolated embryo cells with precipitated protein toxins (fractions 50% concentration, 1.5 to 1.0 mg/mL) where it was found to fail enter into the L1 larvae stage. *C. elegans* embryos treated with toxins showed retarded growth and development; even after 48 hrs of incubation, none of the embryo enters into L1 larvae stage of animal (Table 3). However, isolated embryo cells from the control, entered into the normal L1 larvae developmental stage after 12 hrs of incubation. These experimental data showed that *S. Typhi* toxin protein exposure affects the *C. elegans* fertility, by directly retarding the embryogenesis. It is evident that increasing concentrations of toxin protein significantly ($p < 0.05$) decreased larvae stage formation.

Serial No.	12hrs L1 Development	24hrs L2 Development	48hrs L4 Development
Control	+	+	+
<i>E. coli</i> OP50 bacteria			
50% <i>S. Typhi</i> extracted protein (1.5 mg/mL concentration)	-	-	-
+ <i>E. coli</i> OP50 bacteria			
50% <i>S. Typhi</i> extracted protein (1.0 mg/mL concentration)	-	-	-
+ <i>E. coli</i> OP50 bacteria			
+ = Present -= Absent L1/I2/I4= <i>C. elegans</i> larval stages			

Table 3
In vivo embryo development assay

3.3.2 Regulation of oxidant and antioxidant proteins of *C. elegans*

Several proteins involved in oxidative stress were modulated during toxin exposure, indicating strong involvement in the cell response to toxins exposure. The measurement of extracellular ROS by DFC staining revealed that Typhi toxin protein treated N2 worms have elevated levels of ROS generation. ROS induction was examined at three-time points (12, 24, and 48 hrs) and it was found that ROS generation was high in treated worms compared with that of control samples (Fig. 6A). Furthermore, the H₂O₂ production level was significantly ($p < 0.05$) higher in Typhi toxin protein treated samples compared with that of control for all tested time points provided in (Fig. 6B). The high level of H₂O₂ indicated the role of reactive oxygen species for nematode mortality.

Several upregulated antioxidant proteins viz, superoxide dismutase enzyme (SOD), catalase (CTL), peroxiredoxin (PRDX), peroxidase (SKPO-1), thioredoxin (TRX) and glutamate-cysteinease (GCS) have corroborated the *in vivo* detection of H₂O₂ associated ROS generation that directly leads to accumulation of molecular damage. The estimation of SOD was evaluated in both control and treated samples at three different time points (12, 24, and 48 hrs). The measurement of SOD alone showed significant increase of 2 fold in Typhi toxin protein treated L4 stage animals compared with that of control provided in (Fig. 6C). Quantitative spectrophotometric analysis of catalase activity of L4 stage treated nematodes showed significantly ($p < 0.05$) high catalase activity for 12 and 24 hrs time points compared with that of control provided in (Fig. 6D). In contrast, treated *C. elegans* at 48 hrs showed significantly ($p < 0.05$) decreased catalase activity, which suggested the rescue against H₂O₂ free radicals and other oxidative stresses appear to be decreased. In host cells, protein carbonyls content were also measured to determine the oxidative damage in the worms. The estimation of protein carbonyl contents was evaluated in both control and treated *C. elegans* at 12, 24, and 48 hrs. At three time points compared with that of control (1.8 ± 1.20 nM/mg, 2.3 ± 1.15 nM/mg and 4.8 ± 1.10 nM/mg), treated worms showed significantly ($p < 0.05$) increased carbonyl content level (2.9 ± 1.20 nM/mg, 12.0 ± 1.20 nM/mg and 16.0 ± 1.25 nM/mg) at 12, 24 and 48 hrs, respectively (Fig. 6E). Elevated levels of protein carbonyls could be caused by an increase in protein oxidation or by a decrease in the turnover rate of the oxidized proteins in treated sample.

3.3.3 Regulation of chaperones and stress proteins

Several chaperones (DAF-21, HSP-16.2, GCS-1, STI-1, GSTO-2, PXN-1, TRX-1) have been differentially regulated in treated *C. elegans*. STRING analysis showed direct interaction of HSP proteins with antioxidant (SOD-1, SKPO-1, CTL-1, PRDX-3, SKPO-1, GCS-1) and stress (STI-1 and TRX-1) proteins. Defence against oxidative stress is very much interrelated with network of heat shock or stress-proteins (Espinosa-Diez et al., 2015). To validate the MALDI-TOF/TOF/MS results, the expression of DAF-21 protein was evaluated using Western blotting. The Western blotting results showed downregulation of DAF-21 protein in treated samples compared with that of controls provided in (Fig. 7A). The 3D view of DAF-21 protein (Fig. 7B). STRING analysis of DAF-21 protein showed direct interaction with several MAP Kinase pathway specific proteins (Fig. 7C). Western blotting analysis of these candidates MAP Kinase pathway specific proteins (JNK-1, p38, SGK-1 and HSP-1) were also downregulated compared with that of controls provided in (Fig. 7A).

3.3.4 Regulation of lipid metabolism proteins

Proteins related to lipid metabolism identified, in this study include ADS-1, ZK669.4, SMS-1, BRE-4 and PNG-1. A regulation of these proteins indicated an altered fat storage in living organism. To validate the fat and lipid deposition change in treated *C. elegans*, Oil-Red-O staining was performed. *C. elegans* stained with yellow gold fluorescence are lipid droplets and red fluorescence is phospholipids. Microscopic images reveal the difference in fat storage. A decrease in red staining was observed in treated *C. elegans* compared with that of control. This result suggested the impact of toxin protein on the level of fat molecules in a host system (Fig. 8A). *C. elegans* treated with *S. Typhi* extracted proteins exhibited significant ROS generation provided in (Fig. 6A). Quantifying a lipid peroxidation is an effective way to measure the effect of oxidative damage. Concurrently estimation of *C. elegans* lipid peroxidation for both control and treated at three time points (12, 24, and 48 hrs) showed significantly increased level of TBARS (2-thiobarbituric acid-reactive substances) formation by H₂O₂, which induced lipid peroxidation in treated samples compared with of control (Fig. 8B).

3.3.5 Regulation of ATP energy production proteins

The proteins related to energy metabolism, identified in this study include VHA-13, H28O16.1, OLA-1, MAI-1, LET-754, C29E4.8 and F40F8.1. The VHA-13 encodes V-ATPase; H28O16.1 encodes alpha subunit of mitochondrial ATP synthase. All these enzymes are responsible for aerobic respiration, which is the efficient pathway for metabolic energy. The downregulation of these enzymes likely exhibited an energy deficit which appeared to be one of the reasons for *C. elegans* death. Hence, the total ATP production of *C. elegans* was measured, where the intracellular ATP level was significantly ($p < 0.05$) low in treated samples compared to control, (Fig. 9). The ATP production in intoxicated worms was low, and these results were corroborated well with the ATP generating proteins in this study.

3.3.6 Regulation of cytoskeletal organization proteins

After 12 hrs of treatment with toxin protein, worms were transferred to NGM plates (food source). The worm treated to 500 µg/mL of *S. Typhi* extracted proteins was active and preceded significantly ($p < 0.05$) a normal life span, their body movements and locomotion was also normal. However, worms treated to 1 mg/mL of toxin proteins exhibited a significantly ($p < 0.05$) a mean life span of 60 ± 5 hrs with retarded locomotion and body movements. The worms treated to 1.5 mg/mL of toxin proteins were immobile, produced no progeny and exhibited significantly ($p < 0.05$) a mean life span of 30 ± 5 hrs as provided in (Fig. 10A).

Also, in this study, several identified proteins viz., UNC-54/60/98, MYO-1/2, LMN-1, ZYX-1, ARX-4, IFTA-2 and DEB-1 which are crucial for locomotion and muscle contraction was downregulated. Interestingly, STRING analysis showed close association between these genes as provided in (Fig. 4A). UNC-54 is the myosin heavy chain protein which is essentially expressed in *C. elegans* for locomotion and egg-laying. The downregulated protein MYO-2 and COL-19 were confirmed by the specific *C. elegans* based experiments. The microscopic imaging studies revealed that treated *myo-2* and *col-19*-GFP tagged protein strains showed high fluorescence compared to control strains fed with *E. coli* OP50 (Fig. 10B). An overview of proteins and pathways targeted by *S. Typhi* protein extract in *C. elegans* during exposure are presented in Fig. 11.

4. Discussion

The nematode model, *C. elegans* is well suited for the studies including developmental biology, molecular biology, host-pathogen interactions, neurobiology and hypoxia (Schouest et al., 2009; Marsh et al., 2012; Ren et al., 2010; Kamaladevi et al., 2017). The present study highlighted the impact of bacterial protein extract on *C. elegans* with the specific attention to proteomic alternations induced by the same. This study showed that *S. Typhi* extracted proteins is significantly involved in differential regulation of host *C. elegans* proteome. Based on the densitometry analysis of 2D-GE images, among 477 spots found 95 and 55 spots were significantly downregulated and upregulated, respectively. The combined spectra of MALDI-TOF/TOF/MS were searched against the Swiss-Prot database of *C. elegans* using a MASCOT engine for identification and characterization. The regulated proteins were identified and annotated for their specific biological and molecular functions.

C. elegans regulates a diversified molecular response upon adverse environmental conditions, bacterial infections and physiological stress to promote adaptation for survival. In this study, intoxication has regulated the expression level of HSP (HSP-90, HSP-16.2, HSP-6 and HSP-4) in wild-type N2 worms. This is corroborated with the previous reports (Frydman et al., 2001; Prithika et al., 2016) where it is stated that HSPs provide an immediate response during stress, tissue damage or bacterial infection. It is anticipated that the identified regulation of HSP during *S. Typhi* may act by modulating structurally denatured/ misfolded proteins to retain their native confirmation and degrading the proteins which are not properly refolded as described earlier (Soti et al., 2005; Powers et al., 2010). Many reports are also in agreement about the role of HSP in bacterial infection and immunity (Wang et al., 2017; Prithika et al., 2016; Jeba Mercy et al., 2016; Durai et al., 2014). Collectively, our results suggested that HSP played a vital role in the stress response of *C. elegans* against *S. Typhi* protein extract. Furthermore, the study have found the downregulation of protein DAF-21 and its interaction with proteins including muscle contraction (MYO-1/2), locomotion (UNC-54), energy hydrolysis (LET-754), ATP synthase complex (H28O16.1), stress response (STI-1), serine/threonine kinase activity (KGB-1), oxidoreductase activity (TRX-1) and HSP-16.2 (Fig. 4A). There are certain earlier studies in support of the above finding, which have suggested the important role of DAF-21 in increasing the *C. elegans* immunity against bacterial pathogenesis (Mohri-Shiomi et al., 2008). In addition, the DAF-21 played a key role in the regulation of MAP Kinase pathway by phosphorylating the mitogen activated protein kinase (MPK-1) (Green et al., 2011). In this study, DAF-21 protein showed a significant fold change compared with that of control (Fig. 7B). Since, DAF-21 plays a crucial role in regulating MAP Kinase pathway (Green et al., 2011), the study have explored its associated proteins (JNK-1, SGK-1, p38 and HSP-1) by western blot analysis (Fig. 7A). In addition it is probable that functional loss of DAF-21 might have inhibited MAP Kinase pathway which in turn might be the cause for nematode susceptibility. DAF-2 was appeared to be important molecular player that activates MAP Kinase for rescuing host from toxins (Green et al., 2011). The expression level of antioxidant enzyme verified the oxidative damage in exposed worms. These findings have corroborated the *in vivo* detection of H_2O_2 and ROS generation which directly leads to accumulation of molecular damage in the host cells. Furthermore, upregulated glutathione transferase enzyme families may be involved in xenobiotic detoxification which provided resistance against pathogen in *C. elegans* as described earlier (Lindblom et al., 2006).

C. elegans fat molecule is dynamic in nature; it increases both in size and number during development (Hellerer et al., 2007). In our study, regulation of several molecular players related to lipid metabolism [ADS-1, ZK669.4, SMS-1, BRE-4 and PNG-1] in response to toxin exposure was identified. This suggested that alteration in lipid metabolism occurs in response to intoxication. The ADS-1 (Alkyl-Dihydroxyacetone phosphate synthase) protein is an ortholog of human AGPS (Alkylglyceron phosphate synthase). ADS-1 protein is required for the initial stage of ether lipid biosynthetic pathway (Shi et al., 2016) which is required for the initial stage of ether lipid biosynthetic pathway (Shi et al., 2016). The downregulation of ADS-1 was found in our study which indicates the inhibition of lipid biosynthesis during exposure.

Humans born with mutations in *agps* gene die early because of severe growth and neurological defects (Braverman *et al.*, 2012). The bre-4 gene encodes β -1, 4-N-acetylgalactosaminyl transferase is required for the toxicity of *Bacillus thuringiensis* Cry5B toxin protein (Kho *et al.*, 2011). The galactose- β 1,4-N-acetylglucosamine containing carbohydrate chains are attached with proteins and lipids that binds with the galectins group (Kasai *et al.*, 1996), plays an important role in biological events such as development, immunity and cancer defense (Yang *et al.*, 2008; Boscher *et al.*, 2011). The downregulation of *C. elegans* fat and lipid metabolism proteins appeared to be directly responsible for reduced levels of stored lipids and fatty acids. Oil-Red-O staining of nematodes produced visual patterns of neutral lipids that are representative of biochemical determinations of fat levels (Wahlby *et al.*, 2014; Fouad *et al.*, 2014). The fatty acids play a critical role in modulating lipid/yolk level in the oocytes and regulating reproductive efficiency of *C. elegans* (Chen *et al.*, 2016). In current investigation the significant changes in lipid metabolisms have proven to be closely related with hypersensitivity of the worms towards *S. Typhi* toxin proteins.

Few candidate molecular players namely, LIN-28, UNC-60, SPL-1, MUT, HMP, Y43F4A.1, STI-1, NPP-1, SAS-5, UNC-98, ZYG-1, PIG-1 CED-2 and TDO-2 were regulated during exposure to toxins which are necessary for reproductive events and oocytes development [www.wormbase.com]. Toxin proteins subsequently affected the reproductive events of *C. elegans* whereas control worms didn't show any egg laying defects. In fact, the downregulation of molecular players might be the reason to induce the morphologically degenerated and developmentally abnormal embryos. The downregulation of ATP during toxin protein exposure suggested that the host probably had an energy deficit which could have lead to *C. elegans* mortality.

During the bacterial protein extract exposure, it was observed that UNC-54/60/98, MYO-1/2, LMN-1, ZYX-1, ARX-4, and DEB-1 proteins were downregulated which are necessary for muscle myosin filament assembly, locomotion, depolymerisation, contraction and regulation of actin polymerization. DIM-1 is an immunoglobulin protein essential for maintaining body wall muscle integrity (Rogalski *et al.*, 2003) and localizes in between the dense bodies and the region of the muscle cell membrane (Otey *et al.*, 2005). The DIM-1 can alleviate the locomotion defects caused by toxins (Wang *et al.*, 2017). Regulation of DIM-1 protein directly affects the expression of UNC-54, ZYX-1 and DEB-1 proteins and reduction of these proteins causes severe muscle disruption and paralysis (Etheridge *et al.*, 2012; Rogalski *et al.*, 2003). The protein ZYX-1(Zyxin) acts as muscle mechanical stabilizer and a sensor for muscle cell damage (Lecroisey *et al.*, 2013). The ZYX-1 protein regulates, stabilizes and maintains posterior mechanosensory neuron extension, new synapse formation and growth during larval development (Luo *et al.*, 2014). Our finding supported the role and involvement of the above players during toxin protein extract exposure. Several identified regulated proteins are involved in Na^{2+} and Ca^{2+} voltage gated channels which lead to degradation in the synapses of neurons, immune response pathway and normal cellular process. In addition few uncharacterized proteins (T19C3.6, TPI-1, F52C9.3, F26E4.3, EEED8.2, E02H1.5, F10.D7.3, R166.3 and C05D10.4) were also found to be regulated.

The inadequacy and the high costs associated with mammalian testing reduced the possibility to evaluate the toxicity of a variety of bacterial toxins, environmental chemicals and pollutants. Our study attests the utility of *C. elegans* as an emerging model in toxicological sciences. Our findings reveal that the amount of oxidized proteins increases in several folds which require a highly organised participation of chaperones to rectify the damage in protein conformation. These changes can also occur during aging. In contrast, enhanced antioxidant systems as well as over expression of heat shock proteins lead to longevity. Taken together, our data suggest that altered DAF-21 protein expression, MAPK, JNK signal pathways, ZNK-1, BRE-4 and BRE-5 were observed for the first time to participate in *C. elegans* defence mechanism against *S. Typhi* protein extract, although their detailed functions and mechanisms in stress responses remain ambiguous. This information will help broaden our knowledge on the mechanism of host-toxin interaction.

Abbreviations

used in this resource article

2D-GE, Two Dimensional Differential Gel Electrophoresis, DAF-21, abnormal Dauer Formation (protein), JNK, C-Jun N-terminal Kinase, MAPK, Mitogen Activated Protein Kinase, MALDI-TOF, Matrix assisted laser desorption ionization-Time of Flight, STRING, Search Tool for the Retrieval of Interacting.

Declarations

Author contributions:

DAM, BB and MB designed and performed the experiments and analysed the data. VK helped in proteomic analysis. KB wrote the manuscript in consultation with DAM and other authors.

Ethics approval and consent to participate:

Not applicable

Conflict of interest:

The authors declare that they have no conflicts of interest with the contents of this article. The authors whose names are listed above certify that they have NO known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication:

The content of the manuscript has not been published, or submitted for publication elsewhere. All authors have approved the manuscript for submission.

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Figures

Figure 1

Graph represents the survival of *C. elegans* during extracted protein exposure: A) Killing assays showing the impact of *E. coli* OP50 and *S. Typhi* toxin proteins on wild type *C. elegans*. In liquid killing assays, 50% – 80% Ammonium Sulphate (AS) precipitated *S. Typhi* toxin proteins caused complete killing of *C. elegans* at 36 ± 4 hrs. B) Toxin protein fractions (50% – 80%) digested by Proteinase-K have not killed the nematode. Whereas Proteinase-K untreated protein fractions (50% – 80%) killed the host and confirmed that the proteins are only responsible for the *C. elegans* mortality. C) The *S. Typhi* toxin protein at 1.5 mg/mL, 1.0 mg/mL and 500 µg/mL concentrations killed *C. elegans* significantly ($p < 0.05$) at 36, 65, and 90 hrs respectively, whereas *C. elegans* to *E. coli* OP50 total protein showed normal life span. D) Medium containing AS concentration (200 – 800 mM/mL) was used to check its toxicity against nematode. Medium exceeding the AS concentration at 500 mM/mL is lethal to *C. elegans*. Each experiment was performed in triplicates. *Differences were considered significant at $p < 0.05$.

Figure 2

2D gel electrophoreses images of *C. elegans* proteome. A) The control nematodes fed with *E. coli* OP50. B) *C. elegans* total proteins treated to 1.5 mg/mL of 50% *S. Typhi* protein fraction. The size of IP strip is 18 cm and the pI gradient is from 3 – 10. The experiment was performed in triplicates.

Gene Ontology of low abundance proteins Gene Ontology of high abundance proteins

Figure 3

Gene Ontology analysis using the UniProtKB online tool showed that *C. elegans* regulatory proteins are involved in binding activity, catalytic activity, cell parts, cellular processes, metabolic processes, larval development, reproduction, and locomotion.

A. STRING analysis of Downregulated proteins

B. STRING analysis of Upregulated proteins

Figure 4

Interactome Map using the STRING tool with a medium confidence score (0.400), revealing an interaction between identified protein players which were regulated by 50% *S. Typhi* toxin protein exposure in *C. elegans*. A) STRING analysis of downregulated proteins. B) STRING analysis of upregulated proteins

Figure 5

A) Functional annotations and protein enrichment score of regulated proteins were performed using the DAVID tool. The biological functions which have the highest number of proteins regulated in response to *S. Typhi* protein fraction exposure are cytoskeleton, metal binding, cell adhesion protease and redox processes. B) Physiological function having highest protein enrichment score in responsible *S. Typhi* protein fraction exposure is embryonic development. C) Light microscopic images of *C. elegans* embryos exposed to *S. Typhi* protein extract fraction (concentration 1.5 ± 1.0 mg/mL) in presence of food showed reproductive failure and degenerated embryo formation.

Figure 6

Quantitative analysis of oxidant and antioxidant proteins of *C. elegans* fed with *E. coli* OP50 and treated with *S. Typhi* toxin proteins and *E. coli* OP50 total proteins. A) ROS estimation, B) H2O2 estimation, C) Quantification of SOD activity, D) Quantification of catalase activity and E) Protein carbonyl content estimation. Data were expressed as mean value of three experiments and the error bars represent SD $\pm$ mean (* $p < 0.05$).

Figure 7

A) Western blotting analysis showed downregulation of JNK-1, p38, DAF-21, SGK-1 and HSP-1 at 12 and 24 hrs, detected by using the specific antibodies. The protein expression levels at each time point were normalized with β -actin. B) Proteins analysed by Western blotting showed a close functional regulation interactions with each other identified using the STRING tool. C) DIA analysis represented 3D view of regulated DAF-21 protein *Differences were considered significant at $p < 0.05$.

Figure 8

A) Control N2 and *S. Typhi* protein extract treated worms were stained with Oil-Red- O reagent. B) Lipid peroxidation quantification.

*Differences were considered significant at $p < 0.05$.

Figure 9

Effects of *S. Typhi* toxin proteins on the energy metabolism. Estimation of the total intracellular ATP in *C. elegans* was significantly (* $p < 0.05$) reduced in treated samples compared with that of positive controls (fed with *E. coli* OP50).

Figure 10

A) Microscopic imaging of *S. Typhi* toxin proteins treated myo-2 and col-19 GFP-tagged strain showed high level of fluorescence compared to controls. *Differences were considered significant at $p < 0.05$.

Figure 11

An overview of proteins and pathways activated and targeted by *S. Typhi* toxin in *C. elegans* are presented in this figure. The Salmonella effector proteins SopBA, SopB, SopD, SopE, SopE2, SipA & Sip B enter into the host cell cytosol (Raffatellu et al., 2005). These complex macromolecules employ several mechanisms in a highly regulated manner and manipulate the host cell in various ways. The toxic macromolecules inhibit protein synthesis by ADP-Ribosylation process (Liu and Yu 2015). Some of these toxic macromolecules act on membrane voltage gated channels (VADC) causing depolarisation of mitochondrial potential (O'Rourke, 2007). The VADC blockage inhibits the ADP, Pi and pyruvate to cross into the mitochondrial membranes to generate ATP. The mitochondrial stress generates Reactive oxygen species (ROS) (Mu et al 2019). Salmonella effector proteins reduce the antioxidant defence mechanism of host and make it immune compromised (Mu et al., 2019). HSPs promote the folding of the imported protein to its native conformation (Vabulas et al., 2010). Downregulation of HSPs protein in treated samples might have increased the unfolded proteins in host. HSP-60 is important molecular players in *C. elegans* involved in the activation of the MAP Kinase and UPR^{Mt} (Jeong et al., 2017). Functional loss of HSP-60 regulatory protein causes the downregulation of the MAP Kinase pathway which might be the reason of nematode susceptibility.

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