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

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Abstract

Bacterial effector molecules are crucial infectious agents that can cause pathogenesis. In the present study, the pathogenesis of toxic *Salmonella enterica* serovar Typhi (*S.* Typhi) proteins on the model host *Caenorhabditis elegans* was investigated by exploring the host’s regulatory proteins during infection through the quantitative proteomics approach. Extracted host proteins were analyzed using two-dimensional gel electrophoresis (2D-GE) and differentially regulated proteins were identified using MALDI TOF/TOF/MS analysis. Of the 150 regulated proteins identified, 95 were downregulated while 55 were upregulated. The interaction network of regulated proteins was predicted using the STRING tool. Most downregulated proteins were involved in muscle contraction, locomotion, energy hydrolysis, lipid synthesis, serine/threonine kinase activity, oxidoreductase activity, and protein unfolding. Upregulated proteins were involved in oxidative stress pathways. Hence, cellular stress generated by *S.* Typhi proteins in the model host was determined using lipid peroxidation as well as oxidant and antioxidant assays. In addition, candidate proteins identified via extract analysis were validated by western blotting, and the roles of several crucial molecules were analyzed in vivo using transgenic strains (*myo-2* and *col-19*) and mutant (*ogr-1*) of *C. elegans*. To the best of our knowledge, this is the first study to report protein regulation in host *C. elegans* exposed to toxic *S.* Typhi proteins. It highlights the significance of p38 MAPK and JNK immune pathways.

Introduction

*C. elegans* is a suitable model to investigate the cellular impact of toxic bacterial proteins; its utility in toxicological studies has been well established. Its short generation time, large brood size, conventional biology, and defined innate immune system have made it suitable for toxicological studies [1–3]. A toxicity analysis of *C. elegans* provides whole animal data with intact and metabolically active reproductive, digestive, endocrine, sensory, and neuromuscular systems [4]. Most importantly, it provides a naïve and rapid biological system that can be used to investigate bacterial toxins and host interaction as it naturally feeds on bacteria [5]. Many Gram-negative and Gram-positive bacteria, which are pathogenic to humans, have also been reported to infect *C. elegans* [6]. Bacterial pathogens promote host pathogenesis through prominent virulent factors, including lipopolysaccharide, flagella, pili, proteases, exotoxin A, and exoenzymes [7]. Phenazine toxicity (a virulence factor) through oxidative stress was first identified in *C. elegans*; [8] it was later reported in *Drosophila*, mice, and plants [9]. The viable cultures of *S.* Typhi, *P. aeruginosa*, *K. pneumonia*, and their isolated lipopolysaccharide (LPS) act as powerful immune activators and lethal agents in this nematode model [10, 11]. The lipoteichoic acid of Gram-positive bacteria was reported to be equivalently antigenic to LPS, which elicits an inflammatory response in the host [12].

Past reports confirmed the utility of *C. elegans* as a model organism for the following human pathogens: *Shigella* spp., *Vibrio alginolyticus*, *Proteus* spp., and *S.* Typhi [13–16]. They discovered the molecular players responsible for host defence in *K. pneumonia* and *P. aeruginosa* pathogenesis through the proteomic approach [17, 18].
Salmonella spp. is responsible for millions of infections per year, ranging from food poisoning to life-threatening systemic typhoid fever [19]. S. Typhi’s resistance to antibiotics is an increasing problem, especially in the Indian sub-continent and Southeast Asia [20]. Typhoid resistant to antibiotics is known as multidrug-resistant (MDR) typhoid [21]. Salmonella spp. uses type III secretion systems (T3SS) to deliver bacterial proteins/toxins directly into eukaryotic host cells to amend their cellular functions [22]. In particular, S. Typhi genome encodes ~4500 proteins identified via protein analysis [23]. Toxic proteins are poisonous substances capable of causing disease 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 [24, 25]. In the current study, we investigated the impact of whole protein S. Typhi extract on the translational machinery of C. elegans by employing proteomic approaches. In this context, enriched whole-cell S. Typhi was used to treat C. elegans and, subsequently, 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 a proteomic approach.

Results

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 (Supplementary 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 h (p < 0.05) for the complete killing of C. elegans (Fig. 1A) with the LT₅₀, (time for half to die) of 27 ± 2 h. Ammonium sulphate precipitated protein fractions (50–80%) killed the C. elegans with mean life span 36 ± 5 h, whereas E. coli OP50 extracted protein fractions (50–70%) at same concentration (1.5 mg/mL) did not show any change in 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 h respectively, whereas positive and negative controls did not show 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).

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 (Supplementary 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 h were taken for the analysis. A 2D-GE was deployed to decipher protein regulation in control and treated samples respectively (Fig. 2A, B). The 2D-GE triplicate gels are shown in Supplementary Figure 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 Supplementary Tables 1 and 2. The most downregulated protein in this study was EEED8.2 with the fold change of 12.4. EEED8.2 is predicted to have lipid binding activity in C. elegans [26]. The most upregulated protein in this study was ORC-2 with the fold change of 12.3. The ORC-2 protein is required to assemble the pre-replication complex and is essential to initiate DNA replication [27].

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

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

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**Fig. 2** 2D gel electrophoresis 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 to 10. The experiment was performed in triplicates.

**Fig. 3** Analysis of Gene Ontology categories. Using the UniProtKB online tool for GO analysis showed that *C. elegans* regulatory proteins are involved in binding activity, catalytic activity, cell parts, cellular processes, metabolic processes, larval development, reproduction, and locomotion.
use to terminally differentiate within 12 h 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 h of incubation, none of the embryo enters into L1 larvae stage of animal (Table 1). However, isolated embryo cells from the control, entered into the normal L1 larvae developmental stage after 12 h 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.

**Fig. 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.
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 DCF staining revealed that *S. Typhi* toxin protein treated N2 worms have elevated levels of ROS generation. ROS induction was examined at three-time...
points (12, 24, and 48 h) and it was found that ROS generation was high in treated worms compared with that of control samples (Fig. 6A). Furthermore, the H$_2$O$_2$ 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 H$_2$O$_2$ 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-cysteinase (GCS) have corroborated the in vivo detection of H$_2$O$_2$ 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 h). 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 h time points compared with that of control provided in (Fig. 6D). In contrast, treated C. elegans at 48 h showed significantly ($p < 0.05$) decreased catalase activity, which suggested the rescue against H$_2$O$_2$ 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 h. 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, 12.8 ± 1.20 nM/mg and 16.0 ± 1.25 nM/mg) at 12, 24 and 48 h, 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.

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 [28]. 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 Western blotting protein bands were quantified using the ImageJ software (Fig. 7B). The 3D view of DAF-21 protein showed direct interaction with several MAP Kinase pathway specific proteins (Fig. 7D). 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). Each western blot experiment was performed in biological triplicate (Supplementary Figure 4).

Regulation of lipid metabolism proteins

Proteins related to lipid metabolism identified in this study include EEED8.2, 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). The Oil-Red yellow gold fluorescence was quantified using the ImageJ software, the data showed decreased fluorescence in treated samples compared to control (Fig. 8B). *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 h) 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. 8C). *C. elegans* ogt-1 mutant encodes truncated O-linked N-acetylglicosamine protein that lacks catalytic activity to promote glycosylation, storage of fats and lipid. In liquid killing assay, the L4 stage N2 and ogt-1 mutant worm’s survival rate was monitored (both treated and control) at different time points. The treated ogt-1 mutant worms exhibited significantly (*p* < 0.05) a more life span (mean life span 45 ± 4 h) compared with that of wild type N2 (mean life span 36 ± 5 h). The wild type and mutant worms fed with *E. coli* OP50 food source showed zero mortality and normal life span (Fig. 8D).

**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.

**Regulation of cytoskeletal organization proteins**

After 12 h 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, there 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 h 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 h 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). The myo-2 and col-19-GFP fluorescence was quantified using the ImageJ software; the data showed high fluorescence in treated samples compared to control (Fig. 10C, D). An overview of proteins and pathways targeted by S. Typhi protein extract in C. elegans during exposure are presented in Fig. 11.

**Discussion**

The nematode model, C. elegans is well suited for the studies including developmental biology, molecular biology, host-pathogen interactions, neurobiology and hypoxia [29–32]. The S. Typhi virulence mechanisms were previously investigated using C. elegans as pathogenesis model [33–36]. We have also reported earlier that the C. elegans as a model for S. Typhi infections and provided the preliminary understanding of the nematode infection process [16]. 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. We have used 2D-GE standard technique for comparative proteomics analysis. D-GE visualizes each protein as a spot that can be interpreted by its abundance, location, or even its presence or absence. This technique is capable to resolve up to 10,000 proteins in a single gel from large sets of complex protein mixtures [37, 38]. 2D-GE also provides visual changes in proteins post-translational modifications (PTMs). Resolving a proteins using 2D-GE technique requires close technical attention to every step. In this study 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 levels of HSP (HSP-90, HSP-16.2, HSP-6 and HSP-4) in wild-type N2 worms. This is corroborated with the previous reports [39, 40] 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 [41, 42]. Many reports are also in agreement about the role of HSP in bacterial infection and immunity [40, 43–45]. 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 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 [46]. During *S. Typhi* infection in *C. elegans*, HSP-90 is required for regulatory events. Phosphoproteomics study shows HSP-90 phosphorylation decreased in *C. elegans* during *S. Typhi* infection [47]. 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) [48]. In this study, DAF-21 protein showed a significant fold change compared with that of control (Fig. 7C). Since, DAF-21 plays a crucial role in regulating MAP Kinase pathway [48], 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 [48]. The innate immune defence response is evolutionarily flexible mechanisms that elevate the chance of survival in imminent danger situations. ROS generation is a deadly weapon of various organisms against pathogens. *C. elegans* produce ROS as an immune response to different types of pathogens and acts as a good read out model to elucidate the aspects of redox biology. Unbalanced ROS production causes cell death, affected host elicits ROS-detoxifying enzymes to deal with deleterious effects of ROS and xenobiotic metabolites. In this study several upregulated antioxidant proteins viz, SOD, CTL, PRDX, SKPO-1, TRX and GCS. These antioxidant enzymes have corroborated the in vivo detection of oxidant generation (H2O2 and ROS) in host (Fig. 6). The expression level of these antioxidant enzymes verified the oxidative damage in exposed worms. Furthermore, upregulated glutathione transferase enzyme families may be involved in xenobiotic detoxification which provided resistance against pathogen in *C. elegans* as described earlier [49].
**C. elegans** fat molecule is dynamic in nature; it increases both in size and number during development [50]. In our study, regulation of several molecular players related to lipid metabolism [EEED8.2, ADS-1, DBT-1, 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 neurotransmitter 5-hydroxytryptamine (5-HT) signaling pathway is a potent modulator of **C. elegans** fat content. The deficiency and excess of 5-HT lead to fat accumulation and loss, respectively. RNA-mediated interference (RNAi) study of EEED8.2 shows that inactivation of this gene partially blocked 5-HT-induced fat reduction [51]. The ADS-1 (Alkyl-Dihydroxyacetone phosphate synthase) protein is an ortholog of human AGPS (Alkylglyceron phosphate synthase). Ether lipids are structurally and functionally important components of cell membrane. ADS-1 protein is required for biosynthesis of ether lipid [52]. The **C. elegans** ADS-1 gene (a human homologue AGPS gene) was downregulated in our study which indicates the inhibition of lipid biosynthesis during exposure. Human born with mutations in AGPS gene die early because of severe growth and neurological defects [53]. The *bre-4* gene encodes β-1, 4-N-acetylgalactosaminyl transferase is required for the toxicity of Bacillus thuringiensis Cry5B toxin protein [54]. The galactose-β
1,4-N-acetylglucosamine containing carbohydrate chains are attached with proteins and lipids that binds with the galectins group [55], plays an important role in biological events such as development, immunity and cancer defense [56, 57]. 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 [58, 59]. The fatty acids play a critical role in modulating lipid/ylk level in the oocytes and regulating reproductive efficiency of C. elegans [60]. 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 [61] and localizes in between the dense bodies and the region of the muscle cell membrane [62]. The DIM-1 can alleviate the locomotion defects caused by toxins [45]. 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 [63]. The protein ZYX-1 (Zyxin) acts as muscle mechanical stabilizer and a sensor for muscle cell damage [64]. The ZYX-1 protein regulates, stabilizes and maintains posterior mechanosensory neuron extension, new synapse formation and growth during larval development [65]. 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, F52C9.3, F26E4.3, 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.

Materials and methods

Maintenance of nematode, C. elegans, and bacterial strains

In this study, we used nematodes wild-type N2 Bristol and out crossed strains, ogr-1, myo-2 (EG5568), and col-19 (TP12) obtained from the Caenorhabditis Genetic Centre (MN, USA). Synchronized L4 stage worms used for in vivo exposure experiments were obtained by bleaching, as described previously by [66], and then washed with M9 buffer (3 g KH\(_2\)PO\(_4\), 6 g Na\(_2\)HPO\(_4\), 5 g NaCl and 1 mL of 1 M MgSO\(_4\) to 1 L H\(_2\)O sterilized by autoclaving). They were used for all in vivo exposure experiments. All physiological assays, including short time exposure, were performed using isolated toxin proteins from S. enterica serovar Typhi (MTCC 733), which was purchased from the Microbial Type Culture Collection and Gene Bank (MTCC; Chandigarh, India). All strains were maintained frozen at \(-80^\circ\text{C}\) in peptone solution (2% peptone, 5% glycerol). Frozen bacteria were usually grown on LB plates with 1.5% agar at 37 °C. A single colony was selected from the plate and inoculated in 3 mL LB medium. The overnight culture was diluted (1:20) into 300 mL LB broth with 0.3 M NaCl to increase bacterial invasion.

Ammonium sulphate (AS) fractionation

Soluble proteins from 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 Na\(_2\)HPO\(_4\), and 0.44 g KH\(_2\)PO\(_4\); pH = 7.5), disrupted with an ultrasonicator
Physiological studies during exposure with S. Typhi toxin proteins in C. elegans

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 h. 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).

C. elegans protein sample preparations

After 24 h 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 s 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) 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.

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 h at 100 V; 1 h at 500 V; 1 h at 1000 V; 2 h at 1000–5000 V (gradient); 1 h at 5000 V; 3 h at 5000–10000 V (gradient) and final focussing was done for 2 h at 10000 V. 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) iodoacetamide (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 100 V (200 mA) for 1 h and 150 V (300 mA) for 7–8 h 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 h on rotary agitator. Subsequently, the gels were destained with Milli-Q H2O for 4 h to reduce the background noise.

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 h. After incubation, peptides were extracted with 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 [71, 72].

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 [17].

In vitro embryo development toxicity assay

C. elegans embryos were obtained from gravid adult worms per protocol [73]. 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).

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 [74] 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 [75]. SOD activity of C. elegans cell lysate supernatant (100 µg of protein) was measured as per [76]. Catalase activity of C. elegans was measured as described earlier [77]. C. elegans carbonyl content was measured as per [78]. Lipid peroxidation was determined as described earlier [79]. Each experiment was performed in biological triplicates and the error bars represent the mean ± SD (*p < 0.05).

ATP assay

Total intracellular ATP of C. elegans control and treated samples was measured as described earlier [80]. 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:

\[
\text{ATP(M)} = \frac{\text{Standard RLU} - \text{Balance reagent RLU}}{\text{Unknown RLU}} \times \frac{\text{Amount of ATP standard added}}{C0} \times \frac{C2}{C0}
\]

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...
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. In total, 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 [43]. 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.

Statistical analysis

All experiments were performed independently in triplicate. The images fluoresce intensities were quantified by ImageJ software. 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.

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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.

Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

Consent for publication

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