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# *O*-GlcNAcylation confers protection against *Staphylococcus aureus* infection in *Caenorhabditis elegans* through ubiquitination†

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Glycosylation is one of the most prevalent post-translational modifications in biological systems. In *Caenorhabditis elegans*, *O*-GlcNAcylation has been shown to be actively involved in the regulation of dauer formation and detoxification of toxins secreted by invading pathogens. On this backdrop, the present study is focused on understanding the role of *O*-GlcNAcylation in *C. elegans* during *Staphylococcus aureus* infection using a gel based proteomic approach. Results of time course killing assays with wild-type and mutants of glycosylation and comparison of results revealed an increase in the survival of the *C. elegans oga-1* mutant when compared to wild-type N2 and the *ogt-1* mutant. Increased survival of *C. elegans* N2 upon *S. aureus* infection in the presence of *O*-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosylideneamino) *N*-phenylcarbamate (PUGNAc-an OGA inhibitor) further confirmed the involvement of *O*-GlcNAcylation in protecting *C. elegans* from infection. The two-dimensional gel-based proteomic analysis of the control and *S. aureus* infected *C. elegans oga-1* mutant followed by mass spectrometric identification of differentially expressed proteins has been carried out. The results revealed that key proteins involved in ubiquitination such as Cullin-1 (CUL-1), Cullin-3 (CUL-3), BTB and MATH domain-containing protein 15 (BATH-15), ubiquitin-conjugating enzyme E2 variant 3 (UEV-3) and probable ubiquitin-conjugating enzyme E2 7 (UBC-7) are upregulated. Real-time PCR analysis further confirms the upregulation of genes encoding the above-mentioned proteins which are involved in the ubiquitin-mediated pathways in *C. elegans*. In addition, treatment of *C. elegans* wild-type N2 and the *oga-1* mutant with PUGNAc + suramin and suramin (an ubiquitination inhibitor), respectively has resulted in increased sensitivity to *S. aureus* infection. Hence, it is presumed that upregulation of proteins involved in the ubiquitination pathway could be the key regulatory mechanism responsible for the enhanced survival of the *oga-1* mutant during *S. aureus* infection.

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## 1. Introduction

Host–pathogen interaction is a wide spread mechanism in which a pathogen maintains itself inside the host at a population level which either directs infection or protection depending on the susceptibility of the host, virulence factor, route of transmission as well as environmental factors.<sup>1</sup> *Caenorhabditis elegans* is one of the best preclinical *in vivo* models to study host–pathogen interactions.<sup>2</sup> Understanding host–pathogen interactions in *C. elegans* at the molecular level reveals the nature of infection and uncovers diagnostic and curative targets to overcome harmful infectious diseases and helps to identify target pathways and mechanisms conserved in higher

organisms.<sup>3</sup> *C. elegans* has been used as a successful model system to study the pathogenesis of Gram-positive<sup>4</sup> and Gram-negative pathogens and fungal pathogens<sup>5</sup> as well as the role of toxins, siderophores<sup>6</sup> and other virulence determinants during host–pathogen interactions. *C. elegans* responds to pathogens through an array of immune pathways such as DBL-1/TGF- $\beta$ ,<sup>7</sup> DAF-2/DAF-16,<sup>8</sup> MAP kinase,<sup>9</sup> ERK and toll-like receptors<sup>10</sup> which are shown to be conserved in human and other model systems. It has recently been reported that *C. elegans* can also be used to study virus–host interactions.<sup>11,12</sup> Host–pathogen interaction leads to alterations in both genomic and subsequently in proteomic levels. From the perspective of the OMICs approach, proteomics is an important phase as it reveals the changes in active players in the system.<sup>13</sup> Due to its genetic amenability and availability of mutants, investigation of *C. elegans* at the post-translational level during host–pathogen interaction could pave a way for identification of novel drug targets and immune pathways. Recently, huge attention has been paid to studying the role of different post-translational

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modifications (PTMs) which could regulate the innate immune response of *C. elegans* during host–pathogen interactions *via* modulating the protein–protein interactions.<sup>13</sup> There are more than 200 PTMs that happen in biological systems. Among these, glycosylation is one of the most prevalent PTMs, in which carbohydrate molecules are covalently attached to amino acid functional groups.<sup>14</sup> Glycosylation plays an important role in prognosis and various disease conditions and is considered as a target for therapy response.<sup>15,16</sup> Glycosylated proteins/glycan compositions were used for the development of protein biomarkers.<sup>17</sup> *O*-GlcNAc cycling has been reported to regulate several biological processes in *C. elegans*. For instance, storage of nutrients, dauer formation, longevity,<sup>18–20</sup> protein homeostasis,<sup>21</sup> neuroprotection,<sup>22</sup> glucose stress response,<sup>23</sup> innate immune response<sup>24</sup> and detoxification of toxins secreted by pathogens.<sup>25</sup> Researchers have extensively used *ogt-1* (ok430) and *oga-1* (ok1207) mutant strains to investigate the role of *O*-GlcNAcylation in regulating the above mentioned biological processes. In addition, *O*-GlcNAcylation also modulates the function of the proteasome, chaperone and intracellular calcium level and interacts with other post translational modifications such as phosphorylation and ubiquitination to regulate certain cellular functions.<sup>26</sup> *O*-GlcNAcylation is an intracellular protein modification that links a single *N*-acetylglucosamine moiety to serine and threonine residues in various target proteins. *O*-GlcNAc (*O*-linked  $\beta$ -*N*-acetylglucosamine) is regulated by two key enzymes namely *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) which catalyse the addition and removal of the *N*-acetylglucosamine moiety to the target protein, respectively.<sup>27</sup> *O*-GlcNAc is a reversible regulatory modification which happens often on cytoplasmic and nuclear proteins. Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is the end product of the hexosamine biosynthetic pathway, which assimilates glucose, amino acid, fatty acid, and nucleotide metabolism to synthesize the substrate for *O*-GlcNAcylation.<sup>28</sup> Catalytic activity and substrate specificity of OGT greatly depend on the UDP-GlcNAc level in the cell, making *O*-GlcNAcylation a nutrient responsive process.<sup>29</sup> Hence the UDP-GlcNAc biosynthesis pathway is termed a nutrient responsive/sensitive pathway. *O*-GlcNAcylation has an impact on the nutrient and cellular signalling processes.<sup>30</sup> *O*-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) is a potent inhibitor of *O*-GlcNAc- $\beta$ -*N*-acetylglucosaminidase and is normally used to inhibit OGA, the enzyme which is responsible for the removal of *O*-GlcNAc from target proteins in biological settings to study the effects of elevated *O*-GlcNAc levels in nuclear and cytoplasmic proteins.<sup>31</sup> Increasing the cellular level of *O*-GlcNAc by glucosamine or PUGNAc has been shown to enhance the ubiquitination.<sup>32,33</sup>

*Staphylococcus aureus* is a Gram-positive, versatile, widespread pathogen and also lives as a commensal organism in the human respiratory tract.<sup>34</sup> *S. aureus* causes a range of infections from skin infections to life-threatening diseases such as endocarditis, pneumonia and Toxic Shock Syndrome (TSS).<sup>35</sup> It has been previously shown that, structural proteins, proteins involved in protein folding and energy production were differentially expressed in *C. elegans* upon *S. aureus* infection.<sup>36</sup>

Previously our group has reported the role of the innate immune response of *C. elegans* and its signalling pathways, functional proteins against *Vibrio alginolyticus*,<sup>37</sup> *Pseudomonas aeruginosa*,<sup>38</sup> *Protease mirabilis*<sup>39</sup> and *Klebsiella pneumoniae*<sup>40</sup> using proteomic approaches. However, proteomic analysis involving the study of the role of post translational modifications especially *O*-GlcNAcylation still remains scanty. Against this backdrop, the present investigation is focused on delineating the role/involvement of *O*-GlcNAcylation in *C. elegans* during *S. aureus* infection using two-dimensional gel-based proteomic analysis followed by MALDI-ToF/ToF identification of differentially regulated proteins.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*Staphylococcus aureus* ATCC 11632 and *Escherichia coli* OP50 (obtained from the Caenorhabditis Genetics Center (CGC)) were maintained in tryptic soy agar and Luria Bertani (LB), respectively. Both the cultures were grown at 37 °C in Luria Bertani (LB) broth for killing assay and infections.

### 2.2. *Caenorhabditis elegans* maintenance

*C. elegans* N2 wild-type, and deletion mutant strains RB1169 [*oga-1* (ok1207)] and RB653 [*ogt-1* (ok430) III] were obtained from the Caenorhabditis Genetics Center (CGC), Minnesota, USA and maintained in standard Nematode Growth Medium (NGM) plates.<sup>41,42</sup> NGM plates were seeded with *E. coli* OP50 as a food source and maintained at 20 °C. The age synchronized L4 stage *C. elegans* were used for all the experimental assays. Age synchronized L4 stage *C. elegans* were obtained by bleaching the egg stage hermaphrodite animals with a 1:1 ratio of commercial bleach and 5 M KOH.

### 2.3. Worm liquid-killing assay

Approximately 10 numbers of wild-type N2 and *oga-1* (ok1207) and *ogt-1* (ok430) mutant *C. elegans* were transferred to a 24 well culture plate containing sterile M9 buffer along with *S. aureus* (20% inoculum with OD 600 = 0.1). Then the plates were incubated at 20 °C and the survival of the worms was monitored for every 2 hours until the complete death of the worms. In addition, the killing assay was also carried out in the presence of PUGNAc (0.5 mM). Animals were considered to be dead if there is no response to touch by a platinum loop or there is invisible contraction of the pharynx. *C. elegans* fed on *E. coli* OP50 served as a control.<sup>43</sup>

### 2.4. Colony forming unit assay

*C. elegans* N2, *oga-1* and *ogt-1* mutants were infected with *S. aureus* for 48 h. After preinfection, approximately 10 infected worms were taken and washed thoroughly with M9 buffer containing 1 mM sodium azide to avoid the exclusion of invaded bacteria from the worms' intestine. To the washed worms, 400 mg of 1 mM silicon carbide particles (HiMedia, India) was added and vortexed for 20 min to disturb the worms so that they release the colonized bacterial cells and the



resulting suspension was serially diluted and plated on the Hichrome *S. aureus* agar (Himedia) to determine the colony forming unit (CFU).<sup>43</sup>

### 2.5. Confocal laser scanning microscopic (CLSM) analysis

To confirm the intestinal colonization, *C. elegans* N2, *oga-1* and *ogt-1* mutants were exposed to *S. aureus* for 72 h. After infection, worms were washed thrice with M9 buffer containing 1 mM sodium azide and stained with 0.1% acridine orange solution for 5 min. After staining, the worms were washed twice with M9 buffer to remove left-over stain. Then, the worms were placed on microscopic slides, covered with cover slips and visualized under CLSM [(LSM 710, Carl Zeiss, Germany) (10 $\times$ , dry, EC Plan-Neofluar objective lens with 0.3 NA and 10 $\times$  optical lens)] using 488 nm argon laser to excite the acridine orange and a 500 to 600 nm band pass emission filter for the detection of the fluorescence signal as mentioned in our previously published reports.<sup>39,44</sup> The fluorescence image acquisition was done using Zen 2009 image software (Carl Zeiss, Germany) in which the scan speed was set to 2 s, bit depth 12 and pin hole was set 1 Airy Unit (AU) as the parameters.<sup>45</sup> The gain master, digital gain and digital offset were set constant for each sample. Further, the Transmitted-Photo Multiplier Tube detector (T-PMT) mode was also enabled in the software to acquire the bright field images. The presence of acridine orange fluorescence in the pharyngeal and intestinal region indicates the colonisation of *S. aureus*.

### 2.6. Pharyngeal pumping assay

To analyze the pharyngeal pumping rate of *C. elegans* during *S. aureus* exposure, wild-type N2, *oga-1* and *ogt-1* worms were exposed to *S. aureus* for different time intervals (54, 60, 66 and 72 h) and transferred to NGM plates and their pumping rate was monitored carefully for twenty consecutive seconds. *C. elegans* fed with *E. coli* OP50 were used as a control.<sup>39</sup> In addition, control and *S. aureus* infected worms were observed under bright field microscope at 200 $\times$  magnification (NIKON SMZ, Japan) to observe the pharyngeal damage.<sup>46</sup>

### 2.7. Two-dimensional gel electrophoresis (2-DGE)

2-DGE was carried out in biological triplicate with minor modifications.<sup>40</sup> The total proteins (1 mg) of *C. elegans* mutant *oga-1* fed with *E. coli* OP50 (control) and *oga-1* mutant infected with *S. aureus* for 72 h were purified using a 2D clean-up kit. The resulting pellets were dissolved in urea-thiourea sample buffer and loaded on to the Immobiline DryStrip gel strips (18 cm, non-linear, pH 3–10 cm) which were subjected to rehydration for 12 h at 20 °C. The rehydrated strips were isoelectrically focused using an Ettan™ IPGphor 3 isoelectric focusing system with standard parameters. Then, the strips were reduced and alkylated with dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Then the strips were placed on 12% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) containing a traceable amount of tracking dye bromophenol blue for the separation of the second dimension of the proteins. After electrophoresis, the gels were stained with colloidal Coomassie Brilliant Blue (CBB) G-250 staining for 12 h and destained with

double distilled water and images were captured using a gel scanner.<sup>40</sup>

### 2.8. Image analysis and in gel trypsin digestion of differentially expressed proteins

The processed 2-DGE gel images were analysed using Image Master 2D Platinum 7.0 software (GE Healthcare). The differentially expressed protein spots were subjected to trypsin digestion for MALDI-ToF/ToF analysis. Briefly, protein spots with 1.5 fold down or upregulation were excised from the gel and washed with MilliQ H<sub>2</sub>O and destained with the destaining solution containing 50% acetonitrile (ACN) containing 25 mM ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>) to remove the CBB. Then the destained gels were dehydrated using 100% acetonitrile and vacuum dried. The dehydrated gel plugs were rehydrated with trypsin digestion buffer (10 mM NH<sub>4</sub>HCO<sub>3</sub> in 10% ACN) containing 400 ng of trypsin and incubated in ice for 45 min. After rehydration, gel plugs were overlaid with 40 mM NH<sub>4</sub>HCO<sub>3</sub> in 10% ACN and incubated at 37 °C for 16 h for tryptic digestion. After digestion, the peptides were extracted with extraction buffer containing (0.1% trifluoroacetic acid (TFA) in 60% ACN). The extraction step was repeated twice to increase the yield and pooled peptides were vacuum dried and purified using C18 zip tips (Merck Millipore) as per the manufacturer's directions and the peptides were analysed using MALDI-ToF/ToF (AXIMA Performance, SHIMADZU BIOTECH). The MALDI MS software generated mono isotopic peak list (*m/z* range of 700–3000 Da) was analysed using Swiss Prot (taxonomy-*C. elegans*) database by MASCOT with the typical protein detection constraints.<sup>47</sup>

### 2.9. STRING and gene ontology analysis

Protein–protein interactions (PPI) between the list of regulated proteins identified from 2-DGE were carried out by STRING 10.5 (<https://string-db.org/>) analysis with a medium confidence score of 0.4. The interacting partners and their role in biological activities were identified by Gene Ontology (GO).<sup>37</sup>

### 2.10. Killing assay with ubiquitination inhibitor

To confirm the role of ubiquitination in the survival of *C. elegans* N2, *oga-1* (*ok1207*) and *ogt-1* (*ok430*) mutants during *S. aureus* infection, a killing assay was carried out as mentioned previously in the presence of PUGNac (0.5 mM), suramin (100  $\mu$ M) and a combination of both cullin-RING E3 ubiquitin ligases inhibitor suramin (100  $\mu$ M) + PUGNac (0.5 mM) respectively.

### 2.11. Total RNA isolation, RT-PCR and quantitative PCR analysis

The total RNA was isolated from the *C. elegans oga-1* mutant infected with *S. aureus* and with the respective control *E. coli* OP50 for 72 h by the TRIZOL method according to the manufacturer's instruction and then the isolated RNA was converted into cDNA using MultiScribe™ Reverse Transcriptase enzyme (Applied Biosystems Inc., USA). Quantitative PCR analysis was performed to study the expression of specific protein genes (*cul-1*, *cul-3*, *bath-15*, *uev-3*, *ubc-7* [Table 1]) involved in *C. elegans*



Table 1 List of primers used for Q-PCR analysis

Genes	Primer sequences
<i>cul-1</i> fp	5'-AATCTCGTCTCGAAGGTGATGG-3'
<i>cul-1</i> rp	5'-TATCGGCTGTTGATCATGGA-3'
<i>cul-3</i> fp	5'-GGAATTTGTGGCCCTCTACA-3'
<i>cul-3</i> rp	5'-AAGAGACGTTTGGCGAGGTA-3'
<i>bath-15</i> fp	5'-TTCGCACGAAAGATCAACTG-3'
<i>bath-15</i> rp	5'-CCAAATCCATCGAAACATCC-3'
<i>uev-3</i> fp	5'-TGTAATAATTGATGGACCTGTTGG-3'
<i>uev-3</i> rp	5'-TTTCCATATTTCCCAAGATTTCG-3'
<i>ubc-7</i> fp	5'-TGCATCTCGATTCTTACGA-3'
<i>ubc-7</i> rp	5'-CAACGTTTGCAGGAGATTCA-3'

during *S. aureus* infection using Power SYBR Green PCR Master Mix (Applied Biosystems). The *C. elegans*  $\beta$ -actin gene was used as an internal control. Gene expression levels were calculated by  $2^{-\Delta\Delta ct}$  values. Gene expression analysis was carried out in biological triplicate.<sup>40</sup>

### 2.12. Statistical analysis

All the experiments were conducted in triplicate and the results were analysed using one-way analysis of variance with a statistical significance *p*-value of <0.05, using the SPSS tool (Chicago, IL, USA).

## 3. Results and discussion

### 3.1. Effect of *S. aureus* infection on the survival and pharyngeal pumping of *C. elegans* N2, *oga-1* mutant and *ogt-1* mutant

The availability of *O*-GlcNAcylation mutants such as *oga-1* (*ok1207*) and *ogt-1* (*ok430*) deletion mutant is beneficial for the study of its role during host–pathogen interactions. The liquid killing assay was carried out in the presence of FUDR (5-fluorodeoxyuridine) to inhibit progeny production. L4 stage *oga-1*, *ogt-1* mutants and wild-type N2 *C. elegans* were challenged with *S. aureus* and the complete mortality of N2 and *ogt-1* mutant were observed at 90 and 80 h, respectively. In contrast, a relatively increased survival of the *oga-1* mutant was observed during *S. aureus* infection. Even after 168 h of infection, 20% of *oga-1* mutant worms had survived (Fig. 1a). Thus, the increased survival could be attributed to the *O*-GlcNAcylation. To further confirm the involvement of *O*-GlcNAcylation in the increased survival of the *C. elegans* *oga-1* mutant, wild-type N2 *C. elegans* was infected with *S. aureus* in the presence of PUGNAc (0.5 mM) which is known to inhibit the activity of *O*-GlcNAcase (OGA). Survival of PUGNAc treated wild type nematodes upon *S. aureus* infection was found to be similar to that of *oga-1* mutant. These results further substantiated that the enhanced *O*-GlcNAcylation in the *oga-1* mutant is a possible factor responsible for the increase in survival of *C. elegans* during *S. aureus* infection. The complete killing of wild type *C. elegans* N2 upon infection with *S. aureus* observed in the present study corroborates well with the previous report wherein complete killing of wild-type N2 worms infected with *S. aureus* was observed at 90 h.<sup>43</sup> In addition, to further confirm that

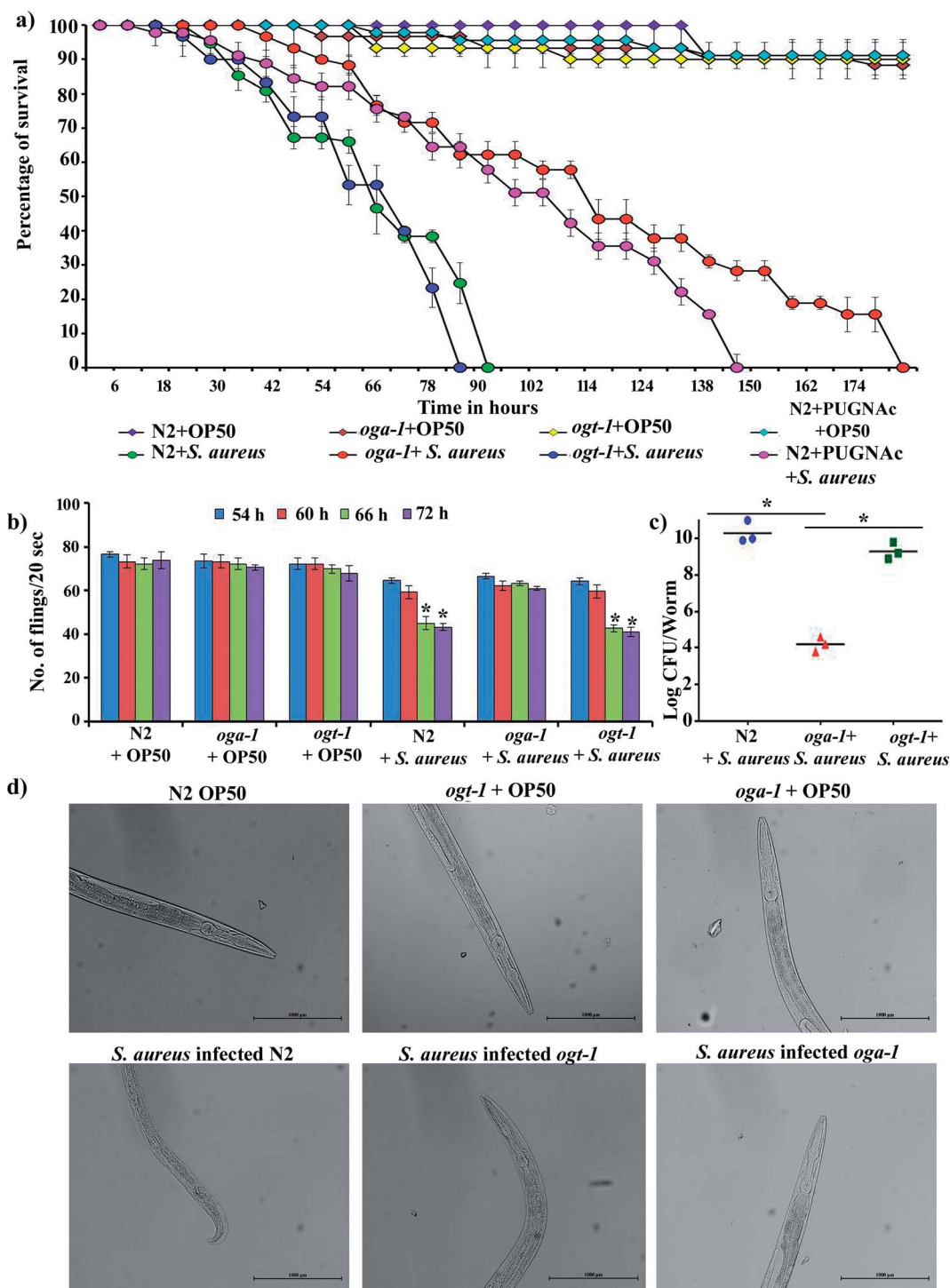
the susceptibility of the *ogt-1* mutant to *S. aureus* infection is due to lack of *O*-GlcNAcylation and not due to lack of nutrition, the killing assay was carried out in the presence of heat killed *E. coli* OP50. Survival of *ogt-1* in the presence and absence of a food source is found to be similar during *S. aureus* infection (ESI Fig. 1†). Hence, it is confirmed that the susceptibility of *ogt-1* mutant to *S. aureus* can be attributed to the lack of *O*-GlcNAcylation. It has been previously reported that *O*-GlcNAc plays a key role in eliciting a pathogen specific immune response in *C. elegans*.<sup>24</sup> Briefly, the *ogt-1* mutant was shown to be highly sensitive to *S. aureus* infection. Whereas, the sensitivity of *C. elegans* wild type and *oga-1* mutant to *S. aureus* NCTC8325 infection was shown to be similar.<sup>24</sup> In contrast to these findings, in the current study we observed the increase in the survival of *oga-1* mutant during *S. aureus* (ATCC 11632) infection. However, in the present study also the *ogt-1* mutant remains highly sensitive to *S. aureus* infection. Merckx-Jacques *et al.*, have shown that the sensitivity of *C. elegans* to environmental *E. coli* isolates differs as a result of variation in the virulence.<sup>48</sup> Recent reports from our group also revealed a difference in survival during infection with different strains of *S. flexneri* (such as *S. flexneri* 2b and *S. flexneri* M9OT).<sup>49,50</sup> In addition, *P. aeruginosa* PA14 has already been reported as more pathogenic to *C. elegans* than *P. aeruginosa* PAO1.<sup>51</sup> Hence, it is hypothesized that the difference in the survival of the *oga-1* mutant against *S. aureus* infection observed in the present investigation when compared to the results of Bond *et al.*, could be attributed to a variation in the virulence of the *S. aureus* strain.

Most Gram-negative and Gram-positive bacteria have been reported to escape from the grinder and antimicrobial peptides present in the *C. elegans* pharynx and colonize in the microvilli brush border and intestine. The pharynx morphology defective mutant (*phm-2*) *C. elegans* is hypersensitive to pathogenic infection.<sup>52</sup> Bacterial pathogens have been known to affect the pharyngeal pumping rate during the course of infection in *C. elegans*<sup>39</sup> and hence, the pharyngeal pumping rate of N2, *oga-1* and *ogt-1* during *S. aureus* infection was observed at 54, 60, 66 and 72 h (Fig. 1b). The results showed profound alterations in the pharyngeal pumping of N2 and *ogt-1* worms upon *S. aureus* infection. Similar to the liquid killing assay, in the *oga-1* mutant, no significant alteration in pharyngeal pumping was observed. When the bacterial exposure time increased, the pharyngeal pumping rate decreased gradually in the N2 and *ogt-1* mutant *C. elegans*. This could be due to damage caused by *S. aureus* in the pharyngeal bulb during infection. These results suggested that the involvement of enhanced glycosylation in the *oga-1* mutant *C. elegans* has prevented the infection caused by *S. aureus* when compared to the wild-type and the *ogt-1* mutant worms.<sup>21</sup> For further confirmation of pharyngeal bulb damage, bright field microscopic analysis was carried out. Light micrographs clearly shows the pharyngeal damage in *S. aureus* infected N2 and *ogt-1* and not in *oga-1* worms (Fig. 1d).

### 3.2. Evaluation of intestinal colonization of *S. aureus* in *C. elegans* N2, *oga-1* mutant and *ogt-1* mutant

Intestinal colonization of bacterial pathogens is one of the important mechanisms required to cause infection under *in*

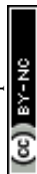




**Fig. 1** Effect of *S. aureus* infection on the survival (a) and pharyngeal pumping (b) of *C. elegans* N2, *oga-1* mutant and *ogt-1* mutant. The CFU assay revealed the reduced intestinal colonization of *S. aureus* in *oga-1* when compared to N2 and *ogt-1* (c). Bright field micrographs of *C. elegans* N2, *oga-1* mutant and *ogt-1* mutant (d) depicting the pharyngeal damage upon infection with *S. aureus*. Error bars represent standard deviations from the mean ( $N = 3$ ). Asterisks indicate the  $p < 0.05$ .

*vivo* conditions. For further confirmation of *S. aureus* intestinal colonization in *C. elegans* N2, *oga-1* and *ogt-1* mutants, a CFU assay was performed. The CFU of *S. aureus* in the N2 and *ogt-1* *C. elegans* after 48 h infection was found to be high, when compared to *oga-1* (Fig. 1c). The intestinal colonization of *S.*

*aureus* in wild type N2 *C. elegans* observed in the present study corroborates well with our previous report.<sup>43</sup> *C. elegans* is a tractable model system to visualize the bacterial intestinal colonization. Hence, to visualize the intestinal colonization of *S. aureus* infected *C. elegans* wild-type N2, *oga-1* mutant and *ogt-1*



mutant are observed under CLSM and the results are presented in (Fig. 2). From the CLSM micrographs, it is evident that the intestinal colonization of *S. aureus* was found to be similar in the N2 and *ogt-1* mutant. Whereas, the wild-type N2+ PUGNac (0.5 mM) and *oga-1* mutant showed reduced intestinal colonization of *S. aureus*.

### 3.3. Proteomic analysis of *C. elegans oga-1* mutant upon *S. aureus* infection

To decipher the molecular mechanism responsible for the increased survival of the *C. elegans oga-1* mutant during *S. aureus* infection 2-DGE was performed and the protein spots were stained with CBB and images were captured and analysed using ImageMaster 2D platinum software to detect the differentially expressed protein spots (Fig. 3). In total 536 spots were matched between control and treated gels and among them, 17

and 22 spots were upregulated and downregulated, respectively (more than 1.5 fold with an ANOVA value of 0.05) and were selected for MALDI-ToF/ToF analysis. A list of differentially identified proteins identified using MALDI-ToF/ToF is presented in Table 2.

Protein-protein interaction networks of the differentially expressed proteins upon *S. aureus* infection in the *oga-1* mutant were predicted using STRING analysis with a medium confidence score of 0.4. Interestingly, the STRING network revealed that the upregulated proteins are able to interact with the proteins involved in the ubiquitin-mediated pathways in *C. elegans* (Fig. 4a). Gene ontology analysis of upregulated proteins revealed their involvement in protein ubiquitination, receptor-mediated endocytosis, vesicle-mediated transport, multicellular organismal development, endocytosis, determination of adult lifespan, multicellular organismal aging, generation of

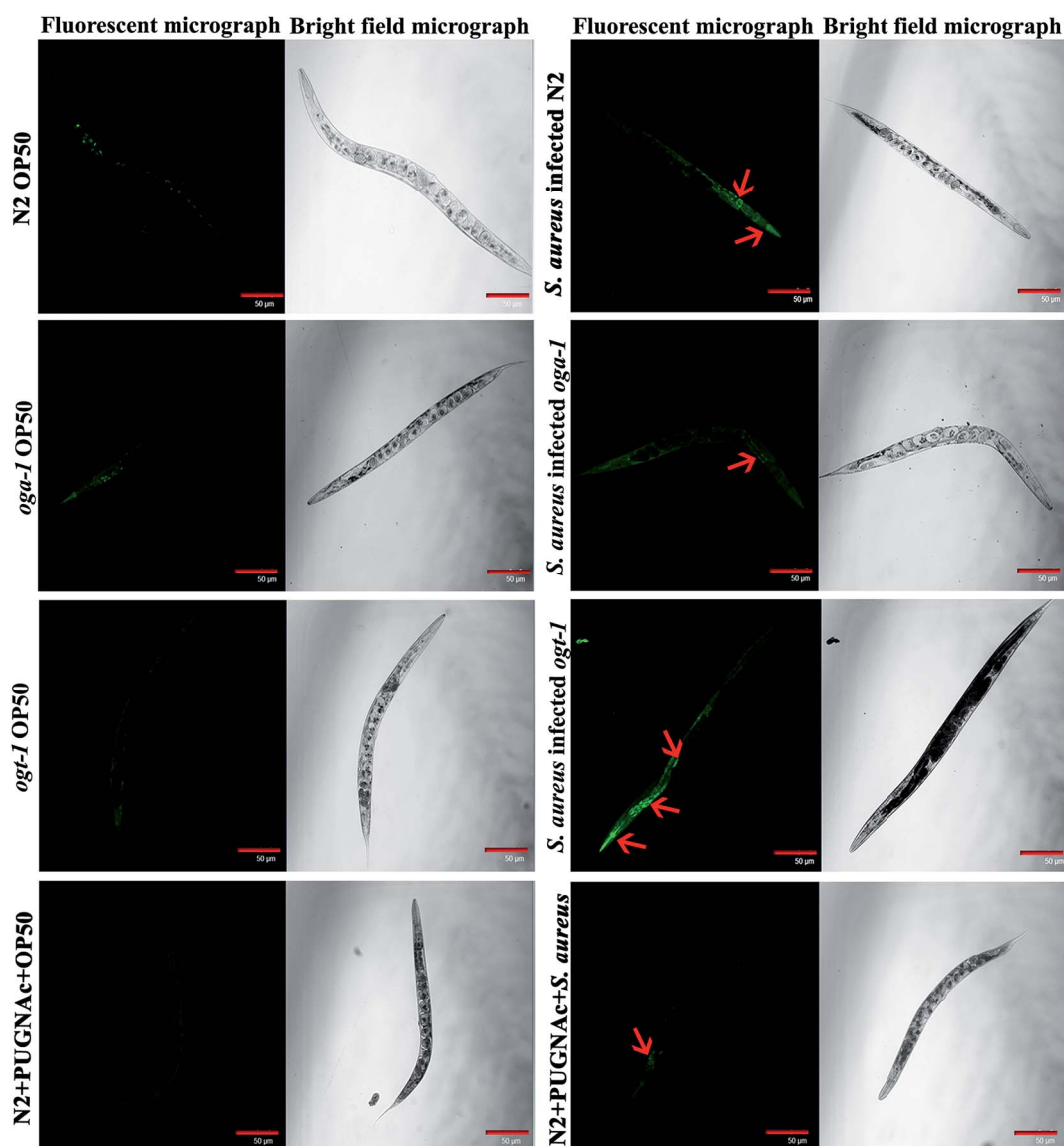


Fig. 2 Representative CLSM micrographs (fluorescence and bright field) depicting the intestinal colonization of *S. aureus* in *C. elegans* N2, *oga-1* mutant and *ogt-1* mutant. Scale bar indicates 50  $\mu$ M.



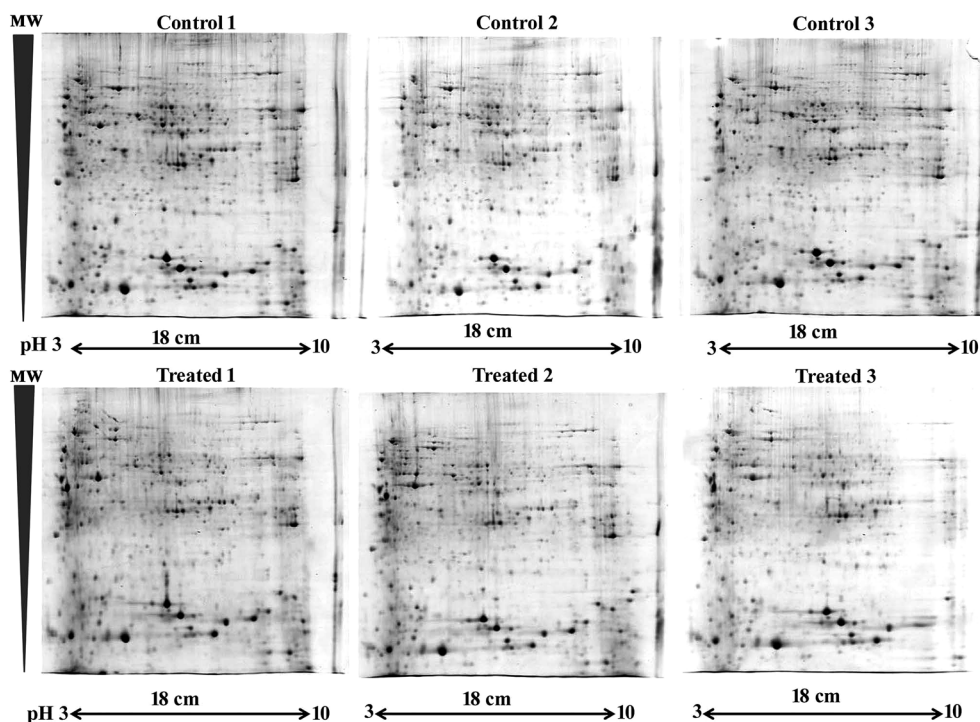


Fig. 3 2D gels depicting the total proteome of *C. elegans oga-1* mutant upon *S. aureus* infection. The upper panel representing the total proteome of *C. elegans oga-1* mutant fed on *E. coli* OP50 and the lower panel representing the total proteome of *C. elegans oga-1* mutant infected with *S. aureus* for 72 h.

precursor metabolites and energy, establishment of localization, anatomical structure development, nematode larval development, cellular metabolic process, embryo development ending in birth/egg hatching, primary metabolic process and single organism reproductive process (Fig. 4b). Whereas, down-regulated proteins are found to be involved in nematode larval development, single-organism developmental process, multicellular organismal development, molecular function, macromolecular complex, respiratory chain, mitochondrial part, mitochondrial inner membrane, protein complex, mitochondrial envelope and intracellular organelle part (Fig. 4c and d).

In the present investigation, proteins involved in the ubiquitination of *C. elegans* such as, Cullin-1 (CUL-1), Cullin-3 (CUL-3), BTB and MATH domain-containing protein 15 (BATH-15), ubiquitin-conjugating enzyme E2 variant 3 (UEV-3) and probable ubiquitin-conjugating enzyme E2 7 (UBC-7) were found to be upregulated upon *S. aureus* infection in the *C. elegans oga-1* mutant when compared to the control. Upregulation of the above-mentioned proteins hinted at enhanced ubiquitination in the *S. aureus* infected *C. elegans oga-1* mutant. Ubiquitination is well known for its involvement in degradation of proteins by the Ubiquitin-Proteasome System (UPS). Previously, it has been reported that a ubiquitination-mediated response is required for the survival of *C. elegans* during Orsay virus and *Nematocida parisii* infection.<sup>53</sup> In eukaryotes, ubiquitin ligases are the group of proteins mainly involved in ubiquitination, which requires the Skp1-Cul1-F-box protein (SCF) to form a multi-subunit RING-finger type ligase.<sup>54</sup> CUL-1 and CUL-3 are the important hydrophobic proteins serve as molecular scaffolds of SCF.<sup>55</sup>

Upregulation of Cullin-6 an ortholog of cullin protein has been shown to be important in *C. elegans* to target the nematode killer *N. parisii* in its intestine. Hence, upregulation of CUL-1 and CUL-3 could be responsible for the enhanced survival of the *C. elegans oga-1* mutant during *S. aureus* infection. F-box and MATH-BTB family proteins are known to have a conserved cullin binding and a variable substrate binding domain.<sup>56</sup> It has already been hypothesized that MATH-BTB proteins are one of the ubiquitin-dependent proteasome adapters that recognize and target foreign proteins, especially proteins, from bacterial and viral origin for proteolysis.<sup>55</sup> Hence, it is presumed that the upregulation of BATH-15 in the *S. aureus* infected *C. elegans oga-1* mutant is responsible for the degradation of proteins involved in the virulence and adherence of the *S. aureus*. Furthermore, it is also hypothesised that the reduction in the intestinal colonization of *S. aureus* in *oga-1* mutant *C. elegans* could also be attributed to the upregulation of proteins involved in the ubiquitination. The possible role of ubiquitination in protecting the *C. elegans oga-1* mutant from *S. aureus* is presented in (Fig. 5).

UEV-3 has been shown to interact with the P38 MAP Kinase family (PMK-3) of the Mitogen-Activated Protein Kinase (MAPK) cascade. Researchers have reported that *Proteus mirabilis*, *Proteus vulgaris* and *Klebsiella pneumoniae* affect the survival of *C. elegans* by targeting the MAPK pathway.<sup>57,58</sup> Hence, the interaction of UEV-3 with PMK-3 is expected to have a role during *S. aureus* infection in the *C. elegans oga-1* mutant.

*C. elegans oga-1* and *ogt-1* mutants have been shown to have reduced fat levels but increased levels of glycogen and trehalose



Table 2 List of differentially expressed proteins identified by MALDI ToF-ToF analysis

S. No.	Regulation	UniProtKB ID	Protein name	Gene name	MASCOT score	No. of peptides matched	Sequence coverage	ANOVA
1	Down	ATPB_CAEEL	ATP synthase subunit beta	<i>atp-2</i>	57	10	30%	0.02
2	Up	GRDN_CAEEL	Girdin homolog	<i>grdn-1</i>	41	9	7%	0.04
3	UP	CUL3_CAEEL	Cullin-3	<i>cul-3</i>	55	13	23%	0.05
4	Down	FAR1_CAEEL	Fatty-acid and retinol-binding protein 1	<i>far-1</i>	61	12	61%	0.04
5	Down	HSP7D_CAEEL	Heat shock 70 kDa protein D	<i>hsp-4</i>	59	9	19%	0.06
6	Up	MDHM_CAEEL	Probable malate dehydrogenase	<i>mdh-2</i>	57	9	30%	0.06
7	Up	CUL1_CAEEL	Cullin-1	<i>cul-1</i>	68	26	29%	0.04
8	Down	COX6A_CAEEL	Probable cytochrome c oxidase subunit 6A	<i>tag-174</i>	55	6	52%	0.00
9	Up	BCAT_CAEEL	Branched-chain-amino-acid aminotranferase	<i>bcat-1</i>	89	19	27%	0.01
10	Up	EPG2_CAEEL	Ectopic P granules protein 2	<i>epg-2</i>	74	28	23%	0.03
11	Up	UCR1_CAEEL	Cytochrome b-c1 complex subunit 1	<i>ucr-1</i>	69	12	33%	0.01
12	Down	ACOX5_CAEEL	Probable peroxisomal acyl-coenzyme a oxidase 5	<i>acox-5</i>	58	12	12%	0.03
13	Down	EI2BG_CAEEL	Probable translation initiation factor eIF-2B subunit $\gamma$	<i>ppp-1</i>	43	4	15%	0.02
14	Down	IPYR_CAEEL	Probable inorganic pyrophosphatase 1	<i>pyp-1</i>	88	11	25%	0.03
15	Down	CYP5_CAEEL	Peptidylprolyl cistrans isomerase 5	<i>cyn5</i>	118	9	29%	0.02
16	Down	RN207_CAEEL	Probable RING finger protein	<i>F47G9.4</i>	47	10	13%	0.05
17	Up	HSP12_CAEEL	Heat shock protein Hsp-16.2	<i>hsp-16.2</i>	29	4	22%	0.02
18	Down	LEC1_CAEEL	32 kDa beta-galactoside-binding lectin	<i>lec1</i>	86	17	48%	0.05
19	Down	VIT6_CAEEL	Vitellogenin-6	<i>vit-6</i>	115	43	26%	0.02
20	Down	CLAP3_CAEEL	Protein CLASP-3	<i>cls-3</i>	35	7	11	0.04
21	Up	TFDP1_CAEEL	Transcription factor dpl-1	<i>dpl-1</i>	52	10	21%	0.04
22	Up	SNB5_CAEEL	Synaptobrevin-like protein 5	<i>snb-5</i>	33	4	29%	0.02
23	Up	ENO_CAEEL	Enolase	<i>enol-1</i>	61	11	31%	0.05
24	Up	BAT15_CAEEL	BTB and MATH domain-containing protein 15 (fragment)	<i>bath-15</i>	59	8	31%	0.02
25	Down	GLS2_CAEEL	Putative glutaminase 2	<i>glna-2</i>	46	11	18%	0.02
26	Down	GPA1_CAEEL	Guanine nucleotide-binding protein alpha-1 subunit	<i>gpa-1</i>	37	7	21%	0.00
27	UP	UB2V3_CAEEL	Ubiquitin-conjugating enzyme E2 variant 3	<i>uev-3</i>	51	12	27%	0.02
28	Up	UBC7_CAEEL	Probable ubiquitin-conjugating enzyme E2 7	<i>ubc-7</i>	35	6	18%	0.02
29	Down	DAF25_CAEEL	Dauer abnormal formation protein 25	<i>daf-25</i>	59	8	13%	0.42
30	Up	GLC7A_CAEEL	Serine/threonine-protein phosphatase PP1-alpha	<i>gsp-1</i>	39	5	18%	0.07
31	Down	NDUAC_CAEEL	Probable NADH dehydrogenase [ubiquinone] 1 alpha sub complex subunit 12	<i>Y94H6A.8</i>	36	3	24%	0.04
32	Up	YQR2_CAEEL	Uncharacterized protein F19C6.2	<i>F19C6.2</i>	54	9	14%	0.04
33	Up	AP2M_CAEEL	AP-2 complex subunit mu	<i>dpy-23</i>	62	8	24%	0.04
34	Down	YNP4_CAEEL	Uncharacterized protein T05G5.4	<i>T05G5.4</i>	57	5	31%	0.49
35	Down	DDX3_CAEEL	ATP-dependent RNA helicase laf-1	<i>laf-1</i>	35	6	9%	0.05
36	Down	KBP3_CAEEL	Kinetochore-binding protein 3	<i>kbp-3</i>	47	5	35%	0.05
37	Up	NDUS2_CAEEL	Probable NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	<i>gas-1</i>	33	5	12%	0.05
38	Down	TBB2_CAEEL	Tubulin beta-2 chain	<i>tbb-2</i>	74	11	18%	0.01
39	Down	HM21_CAEEL	Homeobox protein ceh-21	<i>ceh-21</i>	62	9	22%	0.05

when compared to wild-type N2.<sup>59,60</sup> In the present study, fatty-acid and retinol-binding protein 1 (FAR-1) which is involved in lipid binding was found to be down regulated in the *S. aureus* infected *C. elegans oga-1* mutant. In addition, proteomic analysis revealed the down regulation of Vitellogenin-6 (VIT-6) in the *C. elegans oga-1* mutant upon *S. aureus* infection. *Vit-6* RNAi has been shown to increase the intracellular ROS in *C. elegans*.<sup>61</sup> An elevated level of ROS can cause damage to the nucleic acid, proteins and lipids. However, ROS has also been documented for its ability to modulate the innate immune response. For instance, ROS acts as a signalling molecule for the activation of

nuclear factor kappa B (NF- $\kappa$ B) and MAPK pathway, which are known to play a protective role in *C. elegans* during pathogenic infection.<sup>62</sup>

In addition, proteomic analysis revealed that girdin homolog (GRDN-1) was found to be upregulated upon *S. aureus* infection. GRDN-1 is an ortholog of mammalian girdin and is a novel signalling/scaffolding protein which plays an important role in multiple cellular processes such as division, mobilization and invasion of cells.<sup>63</sup> In *C. elegans*, cilia are expressed ubiquitously and nucleated by the basal body B. Ciliogenesis is the process in which the cilia are assembled or disassembled during the cell





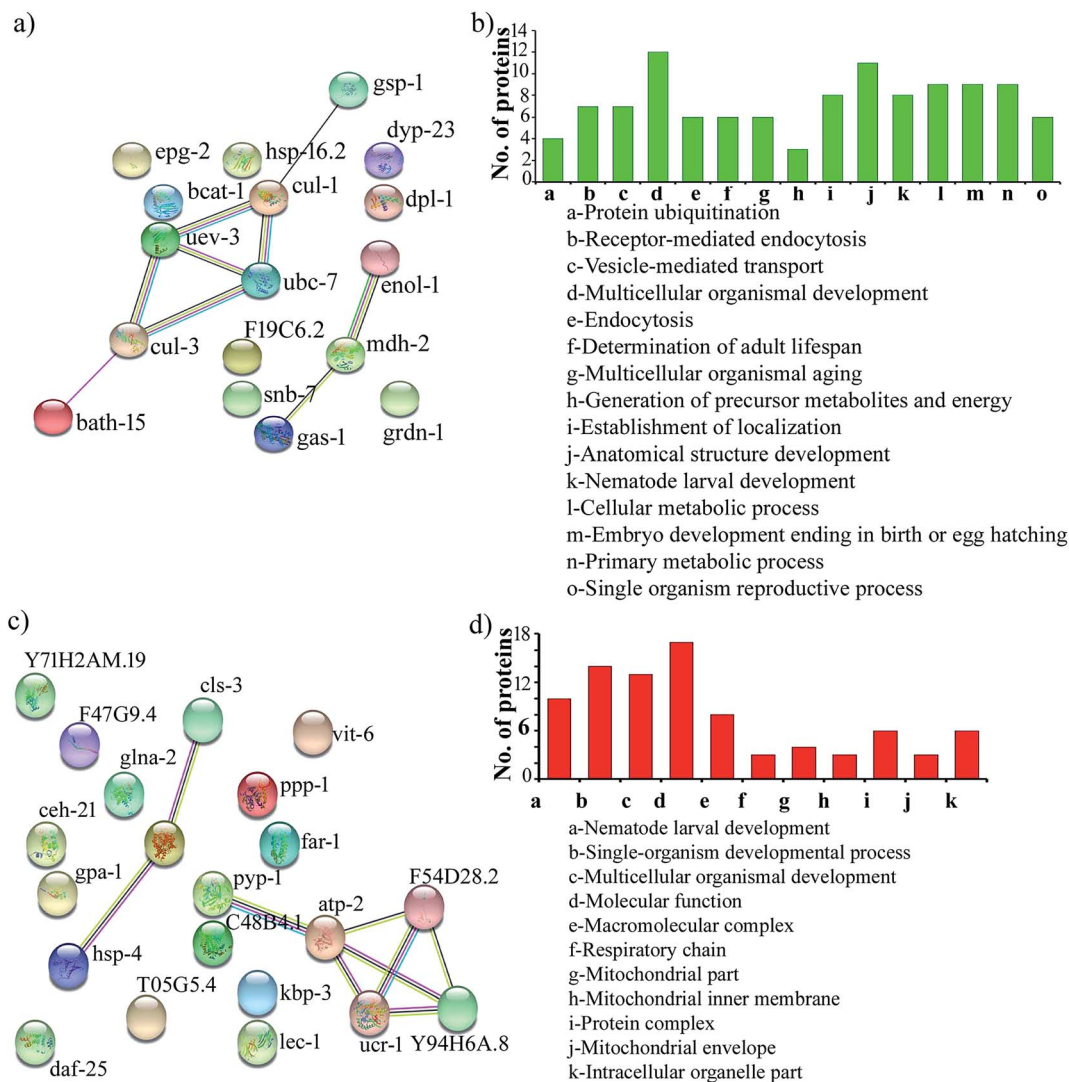


Fig. 4 Protein-protein interaction networks and gene ontology of the upregulated proteins (a & b) and down regulated proteins (c & d).

cycle. Girdin acts as a regulator in positioning the basal body and ciliogenesis in both nematodes as well as in human cells. Upregulation of GRDN-1 in the *C. elegans oga-1* mutant appears to provide the host with more resistance to *S. aureus* infection. Furthermore, ectopic P granules protein 2 (EPG-2) was upregulated in the *oga-1* mutant upon *S. aureus* infection. In *C. elegans*, EPG-2 was involved in the function of autophagosome completion during autophagy.<sup>64</sup> Upregulation of EPG-2 in *oga-1* mutant could also be one of the components responsible for the resistant to *S. aureus* infection.

### 3.4. Effect of PUGNAc and suramin treatment on the survival of *C. elegans* N2 and *oga-1* mutant during *S. aureus* infection

Suramin is already known to inhibit the cullin-RING E3 ubiquitin ligases.<sup>65</sup> Hence, in the present study suramin was used to confirm the role of ubiquitination in increasing the survival of the *oga-1* mutant during *S. aureus* infection. Briefly, *C. elegans* N2 and *oga-1* and *ogt-1* mutants were infected with *S. aureus* in the presence of PUGNAc (0.5 mM), suramin (100  $\mu$ M) and

a combination of PUGNAc + suramin respectively. Interestingly, the susceptibility of *C. elegans* N2 (treated with PUGNAc + suramin) and *oga-1* mutant treated with suramin to *S. aureus* infection was found to be similar. There is no difference in the survival of *oga-1* and PUGNAc treated wild type nematode upon *S. aureus* infection. PUGNAc + suramin treated *ogt-1* mutant worms were found to be more susceptible to *S. aureus* infection. Based on these results, it is confirmed that the upregulation of proteins involved in ubiquitination in the *oga-1* mutant is responsible for its survival during *S. aureus* infection (Fig. 6).

### 3.5. Confirmation of the upregulation of the ubiquitination pathway genes using real-time PCR

The upregulation of genes involved in ubiquitination such as *cul-1*, *cul-3*, *bath-15*, *uev-3* and *ubc-7* are further validated by qPCR analysis using the gene-specific primers mentioned in Table 2. Real time PCR analysis revealed that the mRNA level of the selected candidate genes are found to be upregulated during *S. aureus* infection in the *oga-1* mutant (Fig. 7). These results



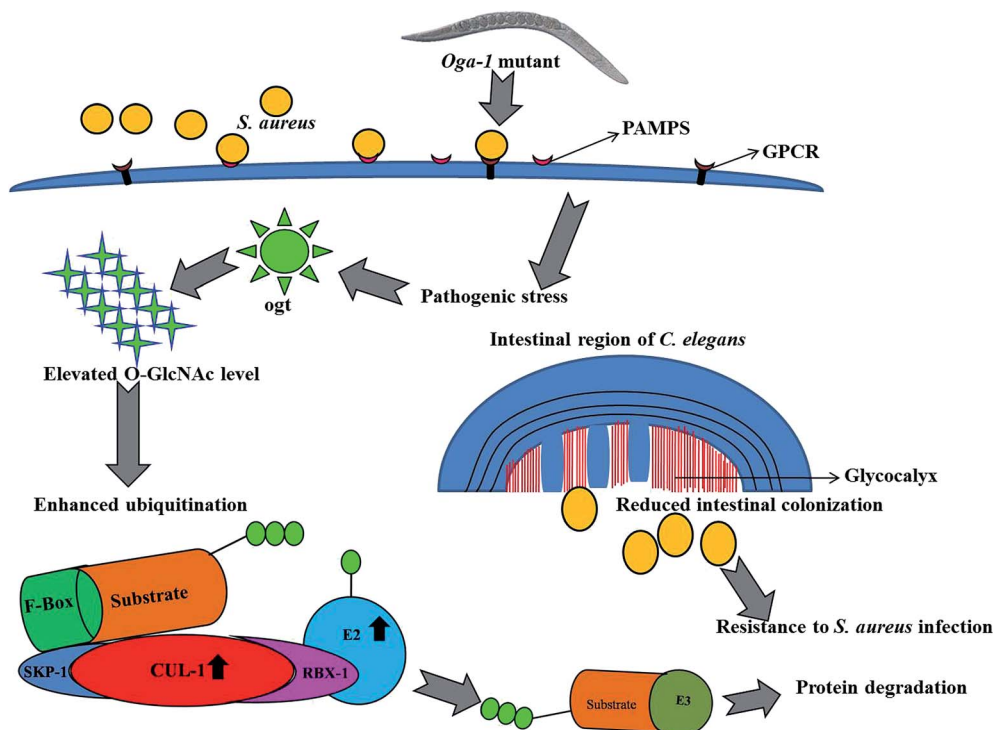


Fig. 5 Schematic representation of outcomes from the present study associates the involvement of ubiquitination and O-GlcNAcylation in the *C. elegans oga-1* mutant during *S. aureus* infection.

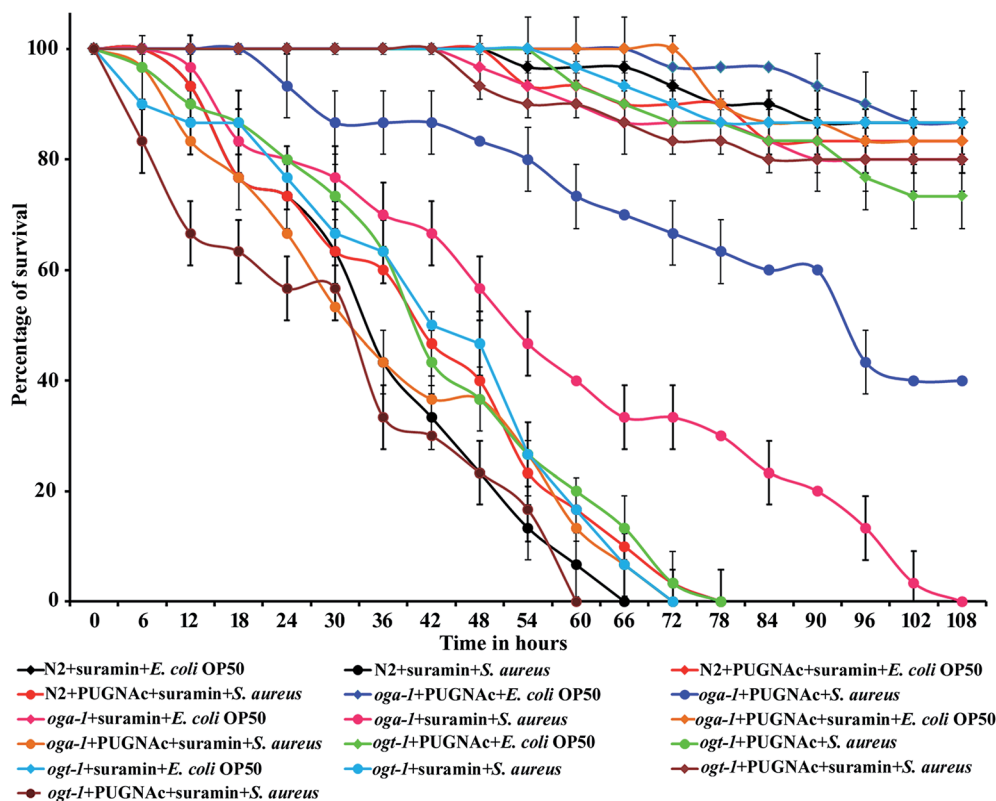


Fig. 6 Effect of PUGNAc, suramin and combination of PUGNAc + suramin treatment on the survival of *C. elegans* N2, *oga-1* and *oga-1* mutant during *S. aureus* infection. Error bars represent standard deviations from the mean ( $N = 3$ ).



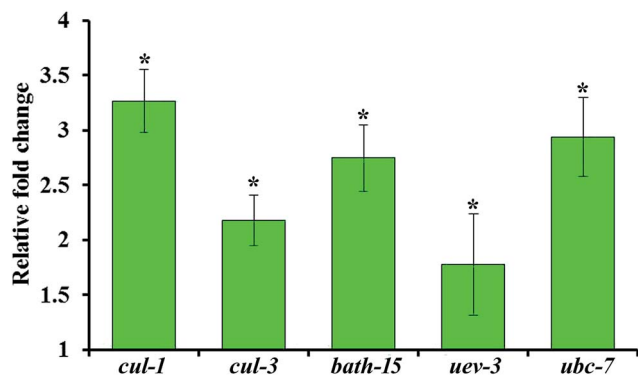


Fig. 7 Real-time PCR analysis of selected genes involved in the ubiquitination pathway in the *C. elegans oga-1* mutant upon *S. aureus* infection. Error bars represent standard deviations from the mean ( $N = 3$ ). Asterisk indicates the  $p < 0.05$ .

further confirm the role of ubiquitination in protecting *oga-1* from *S. aureus* infection.

## 4. Conclusion

In the present study by using *O*-GlcNAcylation specific *C. elegans* mutants, we found that the host acquires more resistance to *S. aureus* infection. Microscopic and CFU assays revealed the decrease in the colonization of *S. aureus* in *oga-1* when compared to the wild-type N2 and *ogt-1* mutant. Treatment of N2 *C. elegans* with PUGNAc, an OGA inhibitor was also found to increase its survival upon *S. aureus* infection. This confirms the role of *O*-GlcNAcylation in protecting *C. elegans* from pathogenic assault. Proteomic analysis was carried out to further confirm the role of *O*-GlcNAcylation during the host–pathogen interaction and the results revealed the upregulation of proteins involved in ubiquitination pathway such as CUL-1, CUL-2, CUL-3, BATH-15, UEV-3 and UBC-7. Real-time PCR analysis revealed the upregulation of *cul-1*, *cul-3*, *bath-15*, *uev-3* and *ubc-7* in *S. aureus* infected *oga-1*, which further substantiates the proteomic analysis results. To further confirm the role of ubiquitination in protecting the *oga-1* mutant from *S. aureus* infection, *oga-1* mutant *C. elegans* was treated with suramin (a known cullin-RING E3 ubiquitin ligases inhibitor) and subsequently infected with *S. aureus*. Suramin treated *oga-1* mutant *C. elegans* was found to be sensitive to *S. aureus* infection. Furthermore, the sensitivity of *C. elegans* N2 treated with a combination of PUGNAc and suramin to *S. aureus* infection was found to be similar to that of untreated *C. elegans*. Based on the results observed in the current investigation, it is hypothesized that *O*-GlcNAcylation protects *C. elegans oga-1* from *S. aureus* infection through upregulation of proteins involved in the ubiquitination pathway.

## Conflicts of interest

The authors declare no conflict of interest.

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