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Proteome Analysis Implicates Adaptive Changes in Metabolism and Body Wall Musculature of *Caenorhabditis elegans* Dauer Larva

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ABSTRACT

Dauer larva is an alternative developmental stage of *Caenorhabditis elegans* (*C. elegans*) that occurs when the environmental condition is unfavorable for growth. Little is known regarding how the proteome of dauer larvae respond to poor environmental growth conditions. Such knowledge is expected to help understand the survival mechanism(s) of dauer larvae. In order to uncover the proteome differences between dauer larvae and normally developed third stage larvae (L3), an L2 stage larvae was starved to create the dauer larvae and this proteome was compared with that of the L3 larvae. Results showed that proteins involved in muscle assembly and fatty acid oxidation are increased in dauer larvae, while proteins involved in maintaining regular organismic activity such as reproduction, translation and apoptotic processes are decreased. The protein expression profile also suggested that the glyoxylate cycle is preferentially utilized during dauer arrest over the tricarboxylic acid (TCA) cycle and significant structural rearrangement occurs on the hypodermis, body wall musculature, and pharynx .

Keywords: larval development, dauer larva, aging, metabolism, proteomics, SILAC

Abbreviations: *C. elegans: Caenorhabditis elegans*; LC-MS: liquid chromatography mass spectrometry; PFOA: perfluorooctanoic acid; SILAC: stable-isotope labeling by amino acids in cell culture; 2D-PAGE: two dimensional polyacrylamide gel electrophoresis.

1. Introduction

Under adequate nutritional and environmental conditions of growth, *Caenorhabditis elegans* (*C. elegans*) rapidly progress from an embryonic stage, through four larval stages (L1 to L4), and to a reproductive adult stage within ~3-4 days at 20 °C. When the L2 larvae encounter unfavorable conditions after progression to the L2 stage (e.g. insufficient food, high temperature, and high-population density), they develop into an alternative L2 stage (L2d) larvae which molt and arrest as dauer larvae until the conditions become favorable [1]. Dauer larvae are thin and surrounded by a specialized cuticle [2]. Their locomotion and metabolic rate are significantly reduced [1,3] and they can survive for several months in this developmentally arrested state without feeding. Once environmental conditions become favorable for growth, the dauer larvae re-enter the life cycle within an hour, and become an L4 stage larvae in approximately 10 hours [4].

Extensive investigation has been done to understand the signaling pathway of dauer formation using molecular genetics and gene expression analysis [3,5,6]. These studies identified many dauer larva formation (daf) genes and revealed that insulin/IGF-1, TGF-β and cGMP signaling pathways have critical roles in dauer formation. However, relatively few proteomic studies have been reported [7–9]. Previous proteomic studies revealed that proteins involved in fatty acid degradation (e.g., alcohol dehydrogenase and aldehyde dehydrogenase) [7] and stress responses (e.g., heat

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shock proteins and antioxidative enzymes) [8,9] are highly expressed in dauer larvae. Although these preceding studies using 2D-PAGE or label-free LC-MS based approaches implied several underlying strategies that dauer larvae take to cope with environmental stress, less than 30 proteins were found to be altered in these studies. Therefore, our knowledge regarding proteome responses to environmental stress remain limited.

In this study, we compared the expression levels of individual proteins in dauer and L3 larvae by a quantitative shotgun approach employing a stable-isotope labeling by amino acids in cell culture (SILAC) [10]. The SILAC approach provided accurate quantitative results on more than 1300 proteins from dauer and L3 larvae. We found that the expressions of many of these proteins were altered in dauer larvae. The observed proteome changes appear to make sense with the alternations in the energy utilization pathways and morphological changes that are known to occur in dauer larvae.

2. Material and Methods

2.1. Materials

$^{12}C_6$- and $^{13}C_6$-lysine (Lys) were purchased from Sigma-Aldrich (St. Louis, MO) and Cambridge Isotope Laboratories (Tewksbury, MA), respectively. Lys-C was purchased from Wako USA (Richmond, VA). All other commercially available chemicals were reagent grade or higher.

2.2. Labeling Bacteria with Light ($^{12}C_6$)-Lys and Heavy ($^{13}C_6$)-Lys

Arginine and lysine auxotrophic *Escherichia coli* (*E. coli*) strain AT713 were obtained from the E. coli Genetic Stock Center at Yale University, and they were labeled with either light ($^{12}C_6$)- or heavy ($^{13}C_6$, 99 atom %)-Lys as described previously [11].

2.3. C. elegans: Strain, Maintenance, and Age Synchronization

WT Bristol N2 strain nematodes were used in this study [12]. Worms were cultured on peptone-free nematode growth media (NGM) plates (51 mM NaCl, 25 mm K_3PO_4, 5 μg/mL cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) seeded with the light ($^{12}C_6$)- or heavy ($^{13}C_6$)-Lys labeled *E. coli*. Detailed protocols for the culture have been described [11]. To synchronize the worms' age, gravid nematodes were treated with a bleach solution (5% sodium hypochlorite solution, 0.25M NaOH) for 3 to 9 minutes, with occasional vortexing. After bleaching, the surviving eggs (embryos) were incubated in liquid M9 media without food and were allowed to hatch as age-synchronized nematodes [11]. The post-hatched period of the egg was counted as the L1 larva.*2.4. Preparation of L3 and dauer larvae*

L3 larvae were prepared by culturing age-synchronized worms on peptone-free NGM plates seeded with light ($^{12}C_6$)-Lys *E. coli* [11] and grown into the L3 larval stage. The larval stage was verified by visual inspection under a microscope. Dauer larvae were prepared by transferring age-synchronized L2 larvae that had been fed with light ($^{12}C_6$)-Lys *E. coli* onto a *E. coli*-free 2% agar plate and culturing them at 20 °C for 48 h. Three plates, each with approximately 500 worms, were prepared for both L3 and dauer larvae. The worms from each plate were pooled and subjected to the following proteomic study.

2.5. Preparation of $^{13}C_6$-Lys labeled reference L3 and dauer larvae

Heavy ($^{13}C_6$)-Lys labeled L3 and dauer larvae were prepared from the eggs laid by the heavy ($^{13}C_6$)-Lys labeled animals and heavy ($^{13}C_6$)-Lys *E. coli* was given as a food source instead of light ($^{12}C_6$)-Lys *E. coli*.

2.6. Sample preparation for proteomic analysis

Light ($^{12}C_6$)-Lys labeled L3 or dauer larvae were resuspended in 250 μL of 100 mM ammonium bicarbonate containing 4% perfluorooctanoic acid (PFOA) (w/v) [13], protease inhibitor mixture (Sigma-Aldrich), and phosphatase inhibitor mixture 3 (Sigma-Aldrich). Proteins were then extracted by ultrasonication (4.5 kHz three times for 9 s with a 3-min pause on ice between the strokes) using a Virsonic 100 ultrasonic cell disrupter (SP Scientific, Warminster, PA) as described previously [10]. The extracted proteins were reduced with 10 mM dithiothreitol (DTT) at 37 °C for 30 min, then *S*-alkylated by 25 mM iodoacetamide at 25 °C for 45 min. Subsequently, the amount was determined with a DC protein assay kit (Bio-Rad, Hercules, CA). A total of 25 μg of protein was digested by Lys-C at a Lys-C: protein ratio of 1:25 (w/w) at 37 °C for 18 hrs. The digest was then mixed with the equal amount of Lys-C digest of heavy ($^{13}C_6$)-Lys labeled L3 and dauer larvae 1:1 mixture, which served as a reference digest, and analyzed by LC-MS/MS as described below.

2.7. LC-MS/MS analysis

LC-MS/MS analysis was carried out using a NanoAcquity Ultrahigh-pressure liquid chromatography system (Waters, Milford, M) interfaced to Velos Pro Ion Trap/Orbitrap Elite Hybrid Mass Spectrometer (Thermo Scientific, Bremen, Germany). Lys-C digests (typically 2 μg) were chromatographed on a reversed-phase 0.075 × 150-mm C18 Acclaim PepMap 100 column (Dionex Inc.) using a linear gradient of acetonitrile from 2% to 37% over 202 min in aqueous 0.1% formic acid at a flow rate of 300 nL/min [14]. The eluent was directly introduced into the mass spectrometer operated in a data-dependent MS to MS/MS

switching mode, with the 25 most intense ions in each MS scan subjected to MS/MS analysis. The full MS scan was performed at a resolution of 120,000 (full width at half-maximum) in the Orbitrap detector, and the MS/MS scans were performed in the ion trap detector in collision-induced dissociation mode. The fragmentation was carried out using the collision-induced dissociation mode with a normalized collision energy of 35 eV. The data were entirely collected in the profile mode for the full MS scan and the centroid mode for the MS/MS scans. The dynamic exclusion function was applied for previously selected precursor ions with the following parameters: repeat count of 1, repeat duration of 40 s, exclusion duration of 90 s, and exclusion size list of 500. Xcalibur software (Version 2.2 SP1 build 48, Thermo-Finnigan Inc., San Jose, CA) was used for instrument control, data acquisition, and data processing.

2.8. Identification and quantification of peptides and proteins

Proteins were identified by comparing all of the experimental peptide MS/MS spectra against the Wormpep database (ftp://ftp.wormbase.org/pub/wormbase/releases) released on May 7 in 2015 using Mascot database search software (Version 2.2.0, Matrix Science, London, UK). Carbamidomethylation of cysteine was set as a fixed modification, whereas variable modifications included oxidation of methionine to methionine sulfoxide, acetylation of N-terminal amino groups, and replacement of C-terminal Lys with heavy Lys. The mass tolerance was set at 10 ppm for precursor ions and 0.8 Da for product ions. Strict Lys-C specificity was applied, and missed cleavages were not allowed. Protein isoforms and proteins that could not be distinguished based on the peptides identified were grouped and reported as a single protein group. The SILAC Quantification Suite in ProteomicsTools software (version 3.9.9, https://github.com/shengqh/RCPA.Tools/releases) [15], was used to determine the abundance of light ($^{12}C_6$)-Lys and heavy ($^{13}C_6$)-Lys labeled proteins, from which the ratio of light protein to heavy protein was calculated.

2.9. Data analysis

All data analyses were performed using RStudio (version 1.0.136) [16]. For each sample, the output of ProteomicsTools software, and the light ($^{12}C_6$)- to heavy ($^{13}C_6$)-Lys labeled protein ratios for individual proteins were transformed to log2 ratios to normalize the data distributions from which the mean log2 ratio of all the proteins were subtracted to reduce the technical variability associated with the experiment. This data processing was done without imputing missing values.

Student's two-sample *t*-test was used to estimate the significance levels for the difference in protein expressions between the dauer and L3 larva groups. A *P*-value of <0.05 with a *q*-value of <0.05 was considered significant. Gene ontology analysis was performed on significantly altered proteins in dauer larvae compared to L3 larvae using DAVID version 6.8 (https://david.ncifcrf.gov/) [17,18].

3. Results

3.1. The expression levels of many proteins are reduced in dauer larvae

Each of the three biological replicates samples from dauer and L3 larvae were analyzed once by LC-MS/MS. All the LC-MS/MS raw data are available from the corresponding author on request. Supplementary Tables S1-6 list all peptides identified and quantified in the data on the dauer and L3 larvae samples. Supplementary Table S7 summarizes all the proteins that were identified and quantified in the study. We were able to identify and quantify 801 proteins from the dauer larvae and 1321 proteins from the L3 larvae, of which 732 proteins were common to both the larval stages, which corresponds to 91% and 55% of proteins identified from the dauer and L3 larva samples, respectively (Fig 1a). The fact that a significantly lesser number of proteins were identified from the dauer larvae may suggest that a substantially smaller number of protein species is required for dauer larvae than for normally developing L3 larvae to maintain their viability.

Since a constant amount of the $^{13}C_6$-Lys reference digest was added to all the digests of the experimental samples prior to the LC-MS/MS, we were able to obtain the light ($^{12}C_6$)-Lys to heavy ($^{13}C_6$)-Lys protein ratios for all the proteins identified. The distributions of log2 Light/Heavy protein ratios for the proteins quantified in each of the triplicate dauer and L3 larvae samples are shown in Fig 1b. The boxplot demonstrates the high reproducibility of the biological replicates. The interquartile ranges (IQR) for the dauer samples (mean IQR on the triplicate samples = 1.18) is

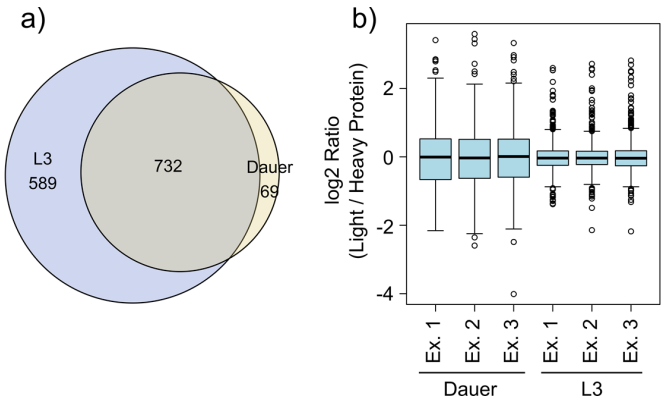

Figure 1. Overview of the proteomics data. (a) Venn diagram depicting the number of proteins identified from the dauer larvae, L3 larvae, and both. (b) Box plot showing the distribution of log2 Light/Heavy protein ratios for the triplicate samples of dauer and L3 larvae. The 95% confidence intervals for the median values on the plots were as follows: dauer Ex. 1 (-0.084 ~ 0.070, n = 0 598), dauer Ex. 2 (-0.105 ~ 0.040, n = 0 610), dauer Ex. 3 (-0.062 ~ 0.080, n = 0 614), L3 Ex. 1 (-0.058 ~ -0.016, n = 0 1039), L3 Ex. 2 (-0.054 ~ -0.017, n = 0 1092), L3 Ex. 3 (-0.062 ~ -0.020, n = 0 1072).

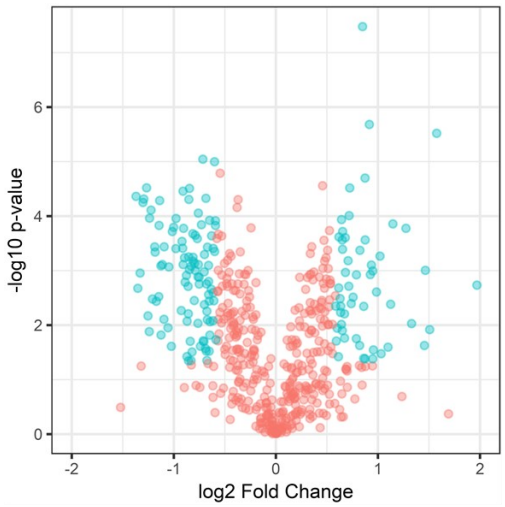

Figure 2. Expression profiles of dauer and L3 larvae proteins. A total of 539 proteins that were quantified in at least two biological replicates in both the dauer and L3 larvae triplicate samples were analyzed. The log2 fold changes (dauer/L3) of proteins are plotted against their negative log10 *p*-values in a volcano plot. Proteins whose expression levels were significantly altered ($p < 0.05$) greater or lesser than 1.5-fold are marked with blue color.

much larger than the L3 larva samples (mean IQR on the triplicate samples = 0.42), indicating that the range of expression levels for individual proteins in dauer larvae is wider than that for proteins in L3 larvae. This suggests that there are higher number of proteins in dauer larvae that are highly up- or down-regulated from those in L3 larvae.

Next we selected 539 proteins that were quantified in at least two biological replicates in both the dauer and L3 larvae triplicate samples. We then plotted the log2 fold changes (dauer/L3) of the 539 proteins against their −log10 *p*-values to visualize the significantly altered proteins (blue dots) (Fig 2). The volcano plot shows that there are 155 proteins (blue spots) that were significantly altered (*p*-value < 0.05) greater or lesser than 1.5-fold in dauer larvae compared to L3 larvae. 54 out of the 155 proteins were expressed in dauer larvae in greater amounts than in L3 larvae while the remaining 101 proteins were expressed in lesser amounts. This result indicates that roughly a quarter of the proteins (155/539) were altered in the dauer larvae. Note that since the same protein amounts from the dauer and L3 larvae samples were analyzed and compared, the log2 dauer to L3 larva fold changes provide relative representation of individual proteins in the total proteome of dauer to L3 larvae.

3.2. Proteins involved in maintaining regular organismic activity are decreased in dauer larvae, while proteins involved in muscle assembly are increased

Proteins that were quantified in at least two biological samples either in the dauer or L3 larvae triplicate samples, which includes the 539 proteins described above, were used in subsequent bioinformatic analyses. The dataset contained a total of 1019 proteins, of which 715 proteins were found in both the dauer and L3 larvae samples. Among the 1019 proteins, 54 proteins were expressed 1.5-fold (both *p*- and *q*-value <0.05) greater in the dauer larvae compared to the L3 larvae (log2 ratio > 0.5849). An additional 19 proteins were uniquely found in the dauer larvae (Supplementary Table S8). We assumed that the unique 19 proteins found only in the dauer larvae were expressed in significantly higher levels compared to the L3 larvae, therefore included in the subsequent bioinformatic analyses. On the other hand, 101 proteins were expressed 1.5-fold (both *p*- and q-value <0.05) lesser in the dauer larvae compared to the L3 larvae (log2 ratio < −0.5849). An additional 375 proteins were found only in the L3 larvae (Supplementary Table S9) and were considered to be expressed significantly higher level in the L3 larvae (lesser levels in the dauer larvae). Note that in addition to *p*- and *q*-value, we applied the threshold value of 1.5 to further filter the dataset to minimize false positives. The threshold value was determined by inspecting the fractions of proteins having *q*-values greater than 0.05 at different threshold values. At the threshold value of 1.5, only 4.9% of proteins had the *q*-values greater than 0.05, indicating that the threshold is not overly assertive and appropriate.

The proteins in Supplementary Tables S8 and S9 were subjected to GO term analysis to predict the physiological changes that might have occurred in the dauer larvae to adapt to starvation conditions. As shown in Fig 3a, proteins increased in the dauer larvae were found to be enriched in six GO-biological processes (BP) terms: 1) locomotion, 2) hermaphrodite genitalia development, 3) lipid storage, 4) striated muscle contraction involved in embryonic body morphogenesis, 5) muscle thin filament assembly, and 6) fatty acid beta-oxidation. On the other hand, many proteins decreased in the dauer larvae were enriched in GO- BP terms that represent normal cellular activities (e.g., reproduction, translation, and apoptotic process). These results suggest that in dauer larvae, metabolic activity is significantly reduced, larval development is halted, muscle related functions are altered, and energy utilization is switched to fatty acids. Similarly, the number of GO-molecular function (MF) terms for proteins that are involved in normal cellular activities such as nucleotide binding, protein binding, and oxidoreductase activity were decreased in the dauer larvae (Fig 3b). Only three GO-MF terms were enriched for proteins increased in the dauer larvae. These were 1) actin binding, 2) motor activity, and 3) actin filament binding, thus implying alterations of muscle assembly in dauer larvae. GO-cellular component (CC) terms enriched for proteins that were increased in the dauer larvae were also mostly related to muscle assembly and contraction. This suggests a significant change in muscle assembly, perhaps to protect them against environmental stress for prolonged survival.

Possible protein-protein interactions between proteins increased in the dauer larvae were examined using STRING web-based software [19]. As shown in Fig 4, the analysis revealed three major networks. The first network involved 11

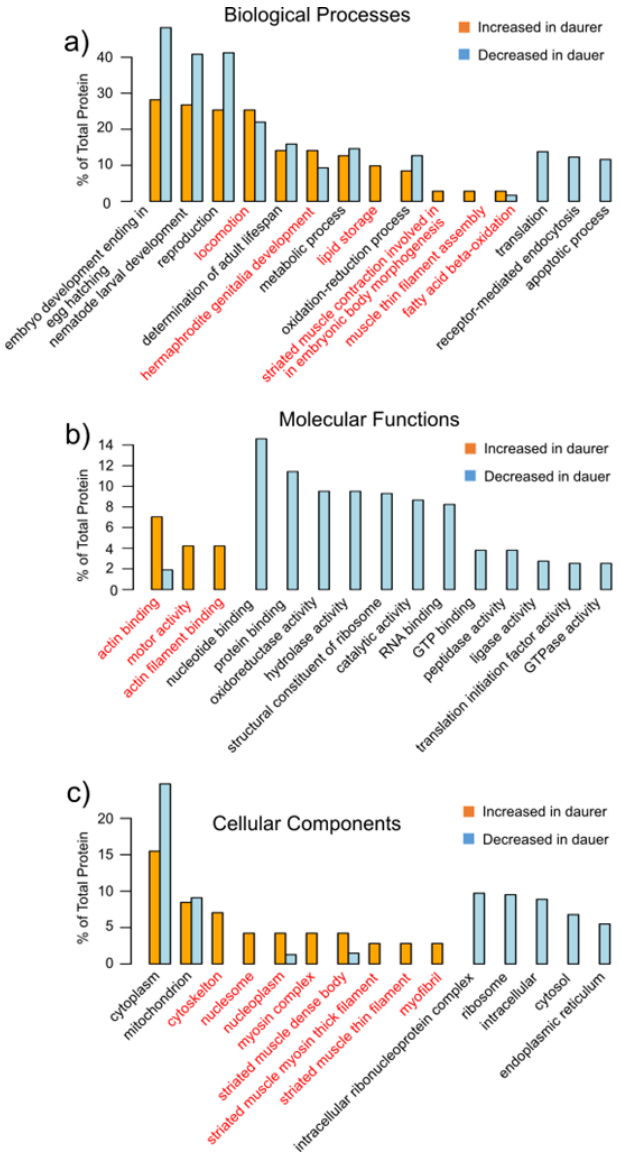

Figure 3. Gene ontology (GO) analysis. The bar plots showing the categorization of the significantly altered proteins (fold-change > 1.5; p-value < 0.05) according to their roles in the major biological processes (a), molecular functions (b) and cellular components (c). The orange and blue bars represent the percentages of the significantly increased and decreased proteins in the dauer larvae from a total of 71 and 473 proteins, respectively. GO terms are ordered from the greatest to least percentages for the significantly increased proteins in the dauer larvae. These are significantly more enriched in dauer larvae than L3 larvae are highlighted in red.

revealed three major networks. The first network involved 11 muscle related proteins, suggesting once again the significant alterations in the muscle assembly of dauer larvae. The second network contained enzymes involved mostly in fatty acid catabolism. The result suggests that the energy utilization in dauer larvae is switched to fatty acids. The third network was comprised of three histone proteins. Increased expression of the histone variants in dauer larvae may suggest regulation of gene expression, cell growth, and proliferation by these histones via regulating DNA accessibility.

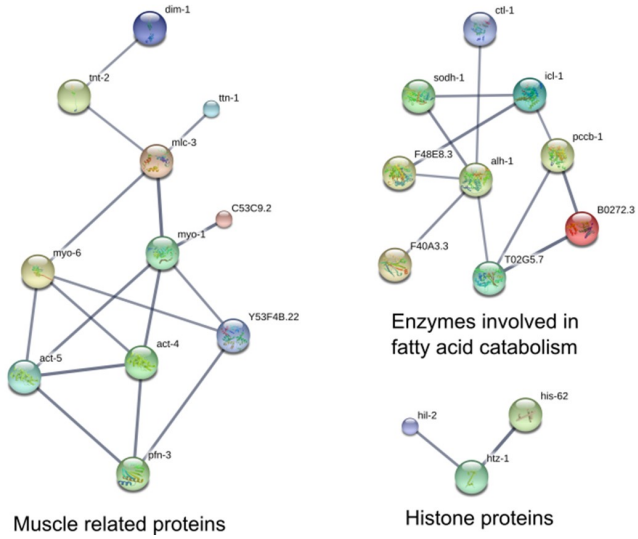

Figure 4. Interaction diagram for the proteins elevated in dauer compared to L3 larvae. The image was created by STRING web software 10.0 with high confidence score > 0.7. Line thickness between nodes indicates the strength of data support. myo-1: myosin-1; myo-6: myosin heavy chain structural genes; mlc-3: myosin essential light chain; pfn-3: profilin-3; dim-1: disorganized muscle protein 1; C53C9.2: uncharacterized protein C53C9.2; tnt-2: troponin T; act-4: actin-4; act-5: actin-5; ttn-1: titin homolog; Y53F4B.22: actin-related proteins; pccb-1: propionyl-CoA carboxylase beta subunit; T02G5.7: uncharacterized protein (possesses acetyl-CoA acetyltransferase activity); B0272.3: probable 3-hydroxyacyl-CoA dehydrogenase; alh-1: aldehyde dehydrogenase; F40A3.3: phosphatidylethanolamine-binding protein homolog; F48E8.3: uncharacterized protein (possesses succinate dehydrogenase activity); icl-1: bifunctional glyoxylate cycle protein; sodh-1: alcohol dehydrogenase 1; ctl-1: catalase-2, htz-1: histone H2A.V; hil-2: histone H1.2; his-62: probable histone H2B 4.

4. Discussion

It is known that the glyoxylate cycle is preferentially utilized in dauer larvae over the tricarboxylic acid (TCA) cycle to convert fat to carbohydrate [20]. There are two glyoxylate cycle specific enzymes, isocitrate lyase and malate synthase (in *C. elegans* the two enzymes are encoded by a single gene and expressed as a bifunctional protein ICL-1), and one TCA cycle specific enzyme, isocitrate dehydrogenase. In our data, the expression level of ICL-1 *(icl-1)* was 1.6-fold higher (p-value < 0.001) and the expression level of NADP-isocitrate dehydrogenase (*idh-1*), inhibition of which was recently shown to promote dauer development [21], were 1.3-fold lower (p-value = 0.014) in dauer larvae compared to L3 larvae. Thus, the results are consistent with the preferential usage of the glyoxylate cycle over TCA cycle in dauer larvae.

Dauer larvae possess a thick protective cuticle that covers the entire body and protects the animal from environmental insults [22,23]. The cuticle is secreted by the epithelial cell hypodermis covering the body. Therefore, it is reasonable to assume that the expression levels of proteins present in the hypodermis are altered. As we predicted, 11 of 73 proteins

Table1. Localization of proteins expressed highly in dauer larvae.

WormBase protein ID	Gene name	UniProt accession number	Protein name	*Expressed location
CE12358	act-4	P10986	Actin-interacting protein 1	body wall musculature, pharynx
CE16463	act-5	O45815	Myosin, essential light chain	pharynx
CE17755	cey-1	O62213	C. elegans y-box	pharynx
CE27706	dim-1	Q18066	Disorganized muscle protein 1	body wall musculature
CE00788	dlc-1	Q22799	Neuronal calcium sensor 2	body wall musculature, pharynx
CE00788	dlc-1	Q22799	Dynein light chain 1, cytoplasmic	body wall musculature, pharynx
CE25238	glrx-10	Q9N456	Glutaredoxin	body wall musculature, pharynx
CE01613	gst-5	Q09596	Major sperm protein 10\ 36\ 56\ 76	pharynx
CE01613	gst-5	Q09596	Probable glutathione S-transferase 5	pharynx
CE16405	hip-1	G5EE04	Uncharacterized protein F08B12.4	hypodermis
CE04890	hmg-11	G5EEL9	HMG	body wall musculature, hypodermis, pharynx
CE23521	icl-1	Q10663	Bifunctional glyoxylate cycle protein	hypodermis, pharynx
CE01341	inf-1	P27639	Eukaryotic initiation factor 4A	pharynx
CE47024	lea-1	H2FLL1	Barrier-to-autointegration factor 1	body wall musculature
CE46912	lea-1	H2FLK7	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	body wall musculature
CE50292	lev-11	NA	Tropomyosin isoforms a/b/d/f	body wall musculature
CE28372	mca-3	Q95XP6	Calcium-transporting ATPase	body wall musculature
CE01236	mlc-3	P53014	Myosin, essential light chain	body wall musculature, pharynx
CE01236	mlc-3	P53014	Myosin, essential light chain	body wall musculature, pharynx
CE06253	myo-1	P02567	Muscle M-line assembly protein unc-89	pharynx
CE23736	NA	Q9N5T2	Uncharacterized protein	hypodermis
CE00852	NA	P41938	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	body wall musculature, pharynx
CE08610	NA	Q8MXD9	Uncharacterized protein	body wall musculature, hypodermis, pharynx
CE00852	NA	P41938	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	body wall musculature, pharynx
CE31529	NA	Q20626	Uncharacterized protein	hypodermis
CE21614	oig-2	Q9XWM1	One IG domain	hypodermis, pharynx
CE00194	pat-2	P34446	Integrin alpha pat-2	body wall musculature, hypodermis
CE00194	pat-2	P34446	Integrin alpha pat-2	body wall musculature, hypodermis
CE32473	paxt-1	Q21738	Partner of xrn-2 protein 1	hypodermis
CE07269	pccb-1	Q20676	Propionyl coenzyme A carboxylase beta subunit	body wall musculature
CE07332	pfn-3	Q21193	Profilin-3	body wall musculature
CE04813	sgt-1	Q21746	Bifunctional glyoxylate cycle protein	body wall musculature, pharynx
CE12212	sodh-1	Q17334	EGF-like domain-containing protein C02B10.3	hypodermis
CE20253	sup-1	Q9XWU2	Protein SUP-1	body wall musculature
CE34313	tnt-2	Q7Z072	TropoNin T	body wall musculature
CE36725	ttn-1	G5EFF0	Titin homolog	body wall musculature
CE03924	unc-78	Q11176	Actin-interacting protein 1	body wall musculature, pharynx
CE30426	unc-89	O01761	Muscle M-line assembly protein unc-89	body wall musculature, pharynx
*The information on the location of the expression was obtained from WormBase (http://www.wormbase.org)				

that were increased in dauer larvae were expressed in the hypodermis (Table 1) suggesting that a significant physiological change occurs in the hypodermis of dauer larvae, presumably to facilitate the production of cuticles. It is also known that dauer larvae are extremely thin [23] and they tend to be motionless [24], thus expected to have significant changes in the body wall musculature. Furthermore, we found that 25 of the 73 proteins that were increased in dauer larvae are expressed in the body wall musculature (Table 1), reflecting the significant structural and physiological rearrangement that occurs on the body wall musculature of dauer larvae. Additionally, the feeding organ pharynx undergoes radial shrinkage and cease pharyngeal pumping during dauer arrest [23]. Among the proteins that were increased in dauer larvae (Table 1), the proteins expressed in the pharynx were also found to be enriched (21 of the 73 proteins). Thus, these results seem to reflect significant morphological changes that occur in dauer larvae.

Several proteomic studies have been done on dauer larvae. Mádi and coworkers prepared dauer larvae by the same way we employed and found that the expressions of four proteins are altered in dauer larvae [7]. They were aldehyde dehydrogenase (*alh-1*), alcohol dehydrogenase-1 (*sodh-1*), phosphatidylethanolamine-binding protein (*F40A3.3*), and pyrophosphatase (*pyp-1*). We found that three of the four proteins (aldehyde dehydrogenase, alcohol dehydrogenase, and phosphatidylethanolamine-binding protein) were also identified in our study. Consistent with the previous study, these three proteins were also increased in dauer larvae in our study. Other two proteomic studies on dauer larvae reported by Jones and coworkers [8] and Erkut and coworkers [9] found that proteins involved in the environmental stress response such as heat shock proteins and superoxide dismutase are increased in dauer larvae. We observed many stress response proteins including eight heat shock proteins (*hsp-12.2*, *hsp-3*, *hsp-110*, *hsp-6*, *hsp-1*, *hsp-4*, *hsp-17*, and *hsp-60*), three superoxide dismutases (*sod-2*, *sod-1*, *sod-1*), two glutathione peroxidases (*gpx-5*, *gpx-2*), and five glutathione S-transferases (*gst-7*, *gst-4*, *gst-5*, *gst-36*, *gst-10*). Contrary to the previous proteomic studies, these proteins were not increased in dauer larvae in our study except glutathione S-transferase 5 (*gst-5*) and catalase (*ctl-1*). The discrepancy between ours and other groups' data could be due to the differences in the protocol employed to generate dauer larvae. Jones and coworkers generated dauer larvae by limiting the available food as we did, but the conditions were different from ours [8]. Erkut and coworkers generated dauer larvae by desiccating nematode at a higher temperature (25 °C). The different results observed by different groups suggest that the proteome of dauer larvae could be dependent on the dauer generation procedure despite all showing similar morphological appearances. Lastly, a global gene expression analysis on dauer larvae has been reported [25]. The study found that genes coding anti-stress proteins such as superoxide dismutase, catalase and heat shock proteins are highly expressed in dauer larvae. This is contrary to our proteomic results. The majority of these anti-stress proteins were not altered in our results. The study also found that the expressions of several histone variants are altered in dauer larvae, suggesting the structural changes of dauer chromatin. This observation is consistent with our results. Thus, it appears that the gene expression data and our proteomic data agree to some extent but not completely. Reasons for this inconsistency include 1) differences in the dauer larvae preparation method between the studies, 2) differences in the extent of transcriptome and proteome coverages (>10,000 transcripts vs ~1,400 proteins), and 3) the fact that mRNA levels do not necessarily reflect the levels of protein expressions [26].

5. Concluding Remarks

The proteome changes we observed in dauer larvae are consistent with the metabolic and morphological changes that occur on *C. elegans* during dauer arrest. Our study provides a foundation for a complete understanding of the adoptive mechanism utilized by this important model organism during unfavorable growth conditions.

6. Supplementary material

Table S1. List of peptides quantified in the dauer larvae experiment 1

Table S2. List of peptides quantified in the dauer larvae experiment 2

Table S3. List of peptides quantified in the dauer larvae experiment 3

Table S4. List of peptides quantified in the L3 larvae experiment 1

Table S5. List of peptides quantified in the L3 larvae experiment 2

Table S6. List of peptides quantified in the L3 larvae experiment 3

Table S7. Proteins identified

Table S8. Proteins highly expressed in dauer larvae compared to L3 larvae and proteins found only in dauer larvae

Table S9. Proteins highly expressed in L3 larvae compared to dauer larvae and proteins found only in L3 larvae

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