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Adaptation of the nematode *Caenorhabditis elegans* to extreme osmotic stress

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Lamitina, S. Todd, Rebecca Morrison, Gilbert W. Moeckel, and Kevin Strange. Adaptation of the nematode *Caenorhabditis elegans* to extreme osmotic stress. *Am J Physiol Cell Physiol* 286: C785–C791, 2004. First published November 26, 2003; 10.1152/ajpcell.00381.2003.—The ability to control osmotic balance is essential for cellular life. Cellular osmotic homeostasis is maintained by accumulation and loss of inorganic ions and organic osmolytes. Although osmoregulation has been studied extensively in many cell types, major gaps exist in our molecular understanding of this essential process. Because of its numerous experimental advantages, the nematode *Caenorhabditis elegans* provides a powerful model system to characterize the genetic basis of animal cell osmoregulation. We therefore characterized the ability of worms to adapt to extreme osmotic stress. Exposure of worms to high-salt growth agar causes rapid shrinkage. Survival is normal on agar containing up to 200 mM NaCl. When grown on 200 mM NaCl for 2 wk, worms are able to survive well on agar containing up to 500 mM NaCl. HPLC analysis demonstrated that levels of the organic osmolyte glycerol increase 15- to 20-fold in nematodes grown on 200 mM NaCl agar. Accumulation of glycerol begins 3 h after exposure to hypertonic stress and peaks by 24 h. Glycerol accumulation is mediated primarily by synthesis from metabolic precursors. Consistent with this finding, hypertonicity increases transcriptional expression of glycerol 3-phosphate dehydrogenase, an enzyme that is rate limiting for hypertonicity-induced glycerol synthesis in yeast. Worms adapted to high salt swell and then return to their initial body volume when exposed to low-salt agar. During recovery from hypertonic stress, glycerol levels fall rapidly and glycerol excretion increases approximately fivefold. Our studies provide the first description of osmotic adaptation in *C. elegans* and provide the foundation for genetic and functional genomic analysis of animal cell osmoregulation.

hypertonicity; glycerol; cell volume regulation

THE ABILITY TO TIGHTLY CONTROL solute and water balance during osmotic challenge is an essential prerequisite for cellular life. Cellular osmotic homeostasis is maintained by the regulated accumulation and loss of inorganic ions and small organic solutes termed organic osmolytes (21, 39). Organic osmolytes are "compatible" or "nonperturbing" solutes and are typically found in concentrations of tens to hundreds of millimolar in the cytosol of all organisms from bacteria to humans (46).

The effector mechanisms responsible for osmoregulatory solute accumulation and loss in animal cells are generally well understood (3, 8, 13). However, major gaps exist in our understanding of how cells sense osmotic stress. For example, the signals and signaling pathways by which animal cells detect volume perturbations and activate volume regulatory mechanisms are poorly defined (6, 7, 31). In addition, relatively little is known about the mechanisms by which cells sense and repair osmotically induced cellular and molecular damage (11, 12, 20).

Genetically tractable and genomically defined organisms such as the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* have provided invaluable insights into the molecular bases of cellular osmoregulation. In *Saccharomyces* for example, mutagenesis, forward genetic analysis, and molecular approaches have demonstrated that hypertonicity-induced accumulation of the organic osmolytes glycerol and trehalose is controlled by the high-osmolarity glycerol (HOG) signaling pathway, a mitogen-activated protein kinase (MAPK) cascade (14).

The nematode *Caenorhabditis elegans* is a model organism that provides many powerful experimental advantages for defining the genes, genetic pathways, and molecular mechanisms that give rise to diverse physiological processes (16). These advantages include a fully sequenced genome, a short life cycle, and forward and reverse genetic tractability. *C. elegans* normally lives in the soil, where it can be exposed to constant and extreme osmotic stress. The purpose of the present study was to characterize the osmoregulatory response of *C. elegans* in the laboratory as a prelude to genetic and molecular characterization of osmoregulatory mechanisms. We demonstrate here that *C. elegans* readily survives and adapts to growth media containing 21–500 mM NaCl. Adaptation to hypertonic conditions is mediated in part by accumulation of the organic osmolyte glycerol. Glycerol accumulation occurs primarily by synthesis. Recovery from hypertonic stress is associated with rapid reduction of glycerol levels and increased glycerol excretion to the external medium. Our studies provide the first description of the osmoregulatory response of *C. elegans* during acclimation to and recovery from hypertonic stress and provide an essential foundation for genetic and functional genomic analysis of animal cell osmoregulation.

MATERIALS AND METHODS

C. elegans strains. The wild-type worm strain N2 var. Bristol was grown on enriched peptone agar plates spread with the *E. coli* bacterial strain NA22. Cultures were maintained at 25°C. At this temperature, worms have a generation time of ~3 days and a life span of ~14–21 days (42).

Preparation of hypertonic growth media. Enriched peptone growth medium contains 21 mM NaCl, 1 mM $MgSO_4$, 24 mM KPO_4, 13 μM cholesterol, 20 g/l BactoPeptone, and 25 g/l agar. The osmolality of this medium was increased by addition of NaCl or sucrose.

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Once poured into petri dishes, water can evaporate from solidified agar. To maintain plate-to-plate consistency in water content, several precautions were taken. After pouring, plates were arranged in a single layer on a lab bench, allowed to cool at room temperature, streaked with NA22 *E. coli*, and incubated at room temperature overnight. Bacteria-seeded plates were wrapped tightly in parafilm and stored at 4°C for no more than 4 wk. When used for worm culture, the plates were inverted and placed inside a plastic container lined with wet paper towels.

Survival studies. Synchronized (22) late L4 larvae and young adults were manually transferred to control and hypertonic agar and maintained at 25°C. Survival was determined after 24 h. Worms were considered to be dead if they did not respond to repeated prodding with a platinum wire.

Volume measurements. Worms on agar growth plates were imaged with a Zeiss Stemi SV 11 dissecting microscope and a video camera (model CCD-100; DAGE-MTI, Michigan City, IN). Images were analyzed with Metamorph imaging software (Universal Imaging, Downingtown, PA). Total body volume was calculated by measuring length and width at the widest point of the animal and by assuming that body shape approximated a cylinder.

Identification and quantification of organic osmolytes. Synchronized young adult animals exposed to control or high-NaCl agar were washed three or four times in control (in mM: 85.6 NaCl, 22 KH_2PO_4, 45.3 Na_2HPO_4, and 1 $MgSO_4$; 295–300 mosmol/kgH_2O) or hypertonic (520–700 mosmol/kgH_2O) M9 buffer. The osmolality of the hypertonic M9 was adjusted with NaCl to match that of the high-NaCl agar the worms were grown on. Washed worms were transferred to ~10 ml of control or hypertonic M9 for 2 h to evacuate gut contents and then washed an additional two times with the same buffer.

Evacuated and washed worms were centrifuged to form a loose pellet. Much of the fluid surrounding the worms was removed, and then ~0.5–1 ml of the pellet was dropped by transfer pipette into liquid N_2. Frozen worm pellets were ground to a powder with a mortar and pestle cooled on dry ice. The powder was rinsed from the mortar into a 15-ml polypropylene tube with three successive 1-ml aliquots of 1 N perchloric acid (PCA) to extract organic solutes and precipitate proteins. After centrifugation, the acid supernatant was removed and neutralized with 5 N KOH containing 61.5 mM K_2HPO_4 and 38.5 mM KH_2PO_4. PCA-precipitated pellets were solubilized with 0.1 N NaOH, and protein content was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL).

Organic osmolytes in neutralized PCA extracts were determined by HPLC analysis with a Sep-Pak C18 column as described previously (45). Glycerol levels were also measured with a commercially available kit (R-Biopharm, Marshall, MI).

Measurement of glycerol excretion. Worms were grown on 200 mM NaCl agar for >4 wk and then evacuated and washed in 520 mosmol/kgH_2O M9 buffer as described in *Identification and quantification of organic osmolytes*. The buffer was filtered away from the worms with a 48-μm nylon filter. Worms were scraped gently from the filter into a 1.5-ml Eppendorf tube with a spatula, and 0.5–0.95 ml of either 520 or 190 mosmol/kgH_2O M9 buffer was added. Tubes were rotated gently for 2 h at room temperature. After centrifugation to pellet the worms, the supernatant was removed for glycerol analysis. Protein content of the worm pellet was determined as described in *Identification and quantification of organic osmolytes*.

Northern blot analysis. The *C. elegans* genome is predicted to contain two glycerol 3-phosphate dehydrogenase (GPD) homologs, K11H3.1 and F47G4.3, of the *S. cerevisiae* gene GPD1. The following primers were used to generate PCR products of these genes from a mixed-stage cDNA library: for K11H3.1 sense GATCTCTAGAGCCAGAATTGTTGGAAGC, antisense GATCGGTACCACATCCGATGGTAGCTTCAC; F47G4.3 sense GATCTCTAGAACGTC GTCGCCACGTCATC, antisense GATCGGTACCATGTATGTCCGACGGAAC. As described in previous studies (2, 35), the 60S acidic ribosomal protein subunit encoding gene *rpa-1* (Y37E3.7) was used as a loading control for Northern blot analyses. An *rpa-1* PCR product was generated using the following primers: sense, CGAACCAAGAACT; antisense, GTCGTCATCGGATTCCTCC.

Purified PCR product from each reaction was labeled with digoxigenin (Dig) with a commercially available kit (Dig DNA Labeling Mix, Roche Diagnostics, Mannheim, Germany). Three to five micrograms of purified PolyA mRNA from synchronized adult worms was separated on a 1.5% formaldehyde-MOPS gel and transferred to a nylon membrane by capillary transfer. Dig-labeled probes were diluted to 15 ng/ml, and hybridization and chemiluminescent detection were performed with a commercially available kit (Dig Luminescent Detection Kit, Roche Diagnostics).

Films were scanned as 16-bit images, and band intensity was quantified with Metamorph imaging software. The ratio of GPD to *rpa-1* band intensity was determined, and hypertonicity-induced changes in GPD expression are reported relative to control.

Statistical analysis. Data are presented as means ± SD or SE. Statistical significance was determined with Student's two-tailed *t*-test. *P* values of <0.05 were taken to indicate statistical significance.

RESULTS

Survival during acclimation to and recovery from hypertonic stress. C. elegans normally lives in soil environments and is likely exposed to extreme osmotic stress through periodic desiccation and rehydration. In the laboratory, nematodes are cultured on agar plates seeded with a lawn of *E. coli*. To determine whether *C. elegans* can survive and adapt to osmotic stress under laboratory conditions, we transferred worms from normal growth agar containing 21 mM NaCl to agar with increasing amounts of NaCl. Survival was monitored 24 h after transfer.

Synchronized young adult worms exhibited >80% survival when exposed to agar containing up to 200 mM NaCl. However, after growing on 200 mM NaCl for 2–4 wk, synchronized young adults survived well on agar containing up to 500 mM NaCl (Fig. 1*A*).[1]

To assess whether hypertonicity or high NaCl concentrations reduce survival, we exposed worms to agar containing 305, 484, or 652 mM sucrose. These concentrations of sucrose were determined by vapor pressure osmometry to be osmotically equivalent to solutions containing 200–400 mM NaCl. On 305 mM sucrose agar, 78 ± 7% ($n = 6$) of the worms were alive after 24 h. This survival was similar to that observed on 200 mM NaCl agar (see Fig. 1*A*). When exposed to agar containing

[1] It should be noted that worms able to survive NaCl levels up to 500 mM are descendants of the synchronized young adult animals exposed originally to 200 mM NaCl growth agar. After 2–4 wk, the original young adults transferred to 200 mM NaCl at 25°C would have given rise to at most four to eight generations of offspring assuming a life cycle (i.e., time from fertilization of an oocyte to development of a sexually mature adult) of 3–4 days. Therefore, it is conceivable that the enhanced ability of acclimated worms to survive NaCl levels >200 mM is due to genetic selection of a population of animals with increased osmotic stress resistance. However, we regard such an interpretation as highly unlikely. Synchronized young adult worms grown on 21 mM NaCl agar all die when exposed to 400 mM NaCl for 24 h (Fig. 1*A*). In contrast, ~80% of worms descended from young adults exposed to 200 mM NaCl for 2–4 wk survive a 24-h exposure to 400 mM NaCl (Fig. 1*A*). This indicates that a subpopulation of unacclimated worms normally resistant to 400 mM NaCl does not exist and therefore cannot be selected for during growth on 200 mM NaCl. We conclude that the ability to survive more extreme hypertonic stress after acclimation to 200 mM NaCl primarily involves physiological adaptations. These adaptations most likely include accumulation of organic osmolytes (see Fig. 3) and activation of genes that protect animals from and repair hypertonicity-induced damage.

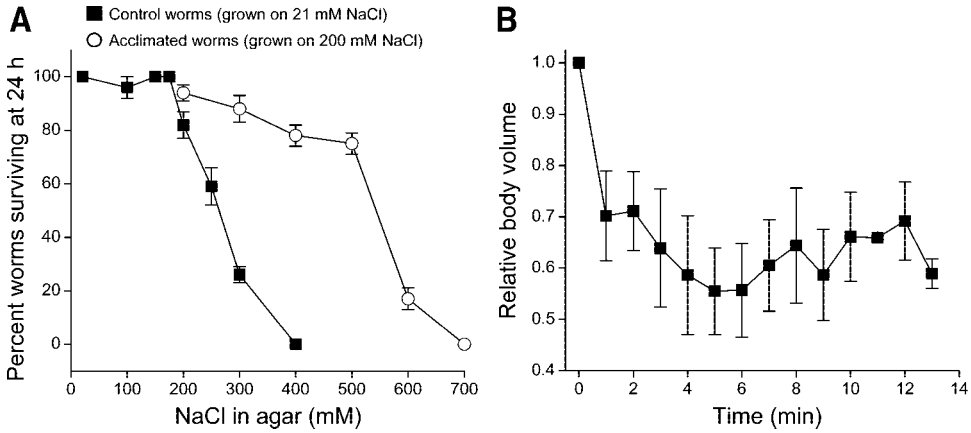

Fig. 1. Effect of hypertonic stress on survival and total body volume. *A*: % of worms surviving 24 h exposure to growth agar containing increasing concentrations of NaCl. Worms were grown on control (21 mM NaCl) agar or acclimated to agar containing 200 mM NaCl for 2–4 wk before exposure to hypertonic conditions. Values are means ± SE (n = 3 or 4 independent experiments). Approximately 10–25 worms were scored in each experiment. *B*: effect of hypertonicity on total body volume. Worms were grown on control agar and then transferred to agar containing 200 mM NaCl. Values are means ± SE (n = 3).

484 and 652 mM sucrose, 59 ± 9% (n = 6) and 21 ± 3% (n = 6), respectively, of the worms survived. These values are somewhat higher than those observed on agar containing osmotically equivalent amounts of NaCl (see Fig. 1*A*). We conclude that hypertonicity reduces worm survival and that survival is further reduced by high concentrations of NaCl.

Hypertonicity-induced water loss from *C. elegans* is expected to occur across the animal's cuticle. *C. elegans* is a filter feeder, and water loss will also occur across the gut as bacteria and associated liquid present on the surface of the agar are ingested by the animal. It is conceivable that the ability to survive extreme hypertonic stress is due to low gut and cuticle water permeability. We examined this possibility by transferring worms to 200 mM NaCl agar and quantifying total body volume over time. As shown in Fig. 1*B*, worms shrank ~40% within 5 min after exposure to hypertonic stress. These results demonstrate that hypertonicity causes rapid water loss and that the cuticle and gut under normal laboratory conditions do not provide significant barriers to dehydration.

We also monitored the ability of *C. elegans* to survive during recovery from hypertonic stress. Worms were grown on agar containing 200–400 mM NaCl for 2 wk and then transferred back to control agar containing 21 mM NaCl. As shown in Fig. 2*A*, survival was nearly 100% 24 h after return to 21 mM NaCl agar.

Hypertonicity-acclimated worms gained water rapidly when transferred to low-salt agar. Total body volume increased ~30% within 5 min when worms acclimated to 400 mM NaCl were returned to 21 mM NaCl agar (Fig. 2*B*). Whole body swelling is followed by fluid loss and return of body volume toward control within ~20 min. Fluid loss is presumably mediated at least in part by the animal's excretory cell or "kidney," as suggested by others (5, 25, 26).

Organic osmolyte accumulation. Accumulation and loss of organic osmolytes represent a universal cellular strategy for surviving osmotic stress. The unique biophysical and biochemical properties of organic osmolytes allow cells to accumulate them to high levels and withstand large shifts in their concentration without deleterious effects on cellular structure and function. In animal cells, organic osmolytes are grouped into three distinct classes: *1*) amino acids, *2*) methylamines, and *3*) polyols (46).

We used HPLC analysis (45) initially to identify organic osmolytes accumulated during hypertonic stress. As shown in Fig. 3*A*, *left*, a number of organic solutes can be detected in worm extracts by HPLC. However, only a single peak (asterisk in Fig. 3*A*) showed consistent and striking increases after worms were exposed to 200 mM NaCl. The elution time of this peak is close to that expected for the organic osmolyte taurine, but amino acid analysis revealed no significant changes in the concentrations of this solute during hypertonic stress (data not shown).

Yeast, fungi, and algae utilize glycerol as a compatible solute when exposed to hypertonicity (14, 36, 47). The nematode *Aphelenchus avenae* accumulates large quantities of glycerol as it undergoes desiccation and entry into an anhydrobiotic state (9, 10). In addition, infective juveniles of the nematode *Steinernema carpocapsae* accumulate glycerol when exposed

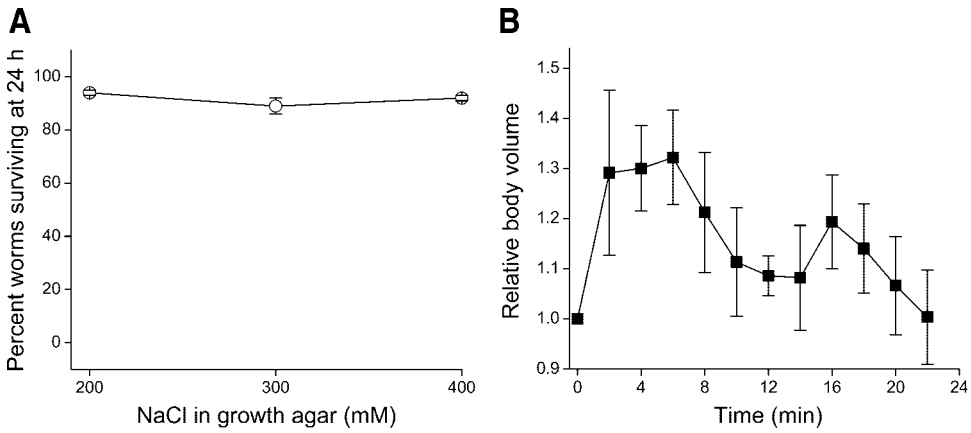

Fig. 2. Survival and total body volume during recovery from hypertonic stress. *A*: % of worms surviving 24-h exposure to control (21 mM NaCl) growth agar after acclimation for at least 1 wk to agar containing 200–400 mM NaCl. Values are means ± SE (n = 3 independent experiments). Ten to twenty-five worms were scored in each experiment. *B*: total body volume during recovery from hypertonic stress. Worms were acclimated to 400 mM NaCl for 1 wk and then transferred to control agar. Values are means ± SE (n = 5).

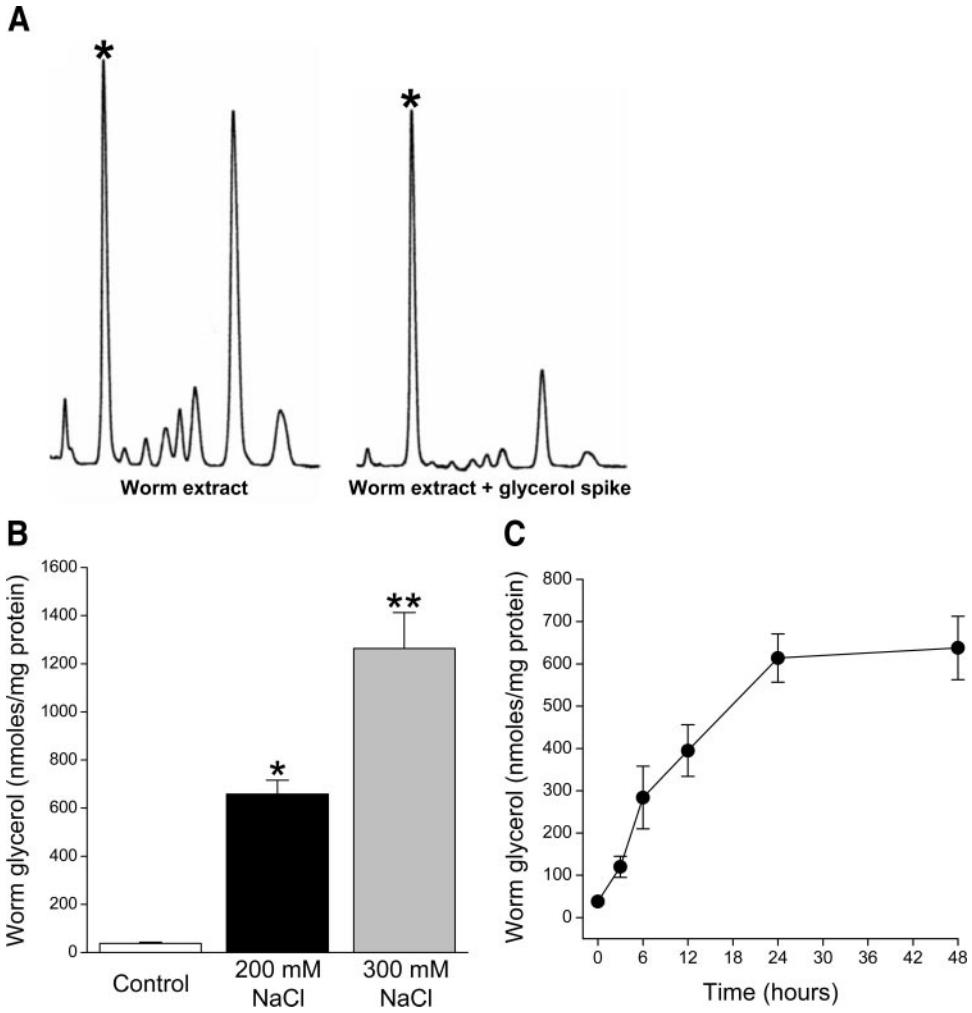

Fig. 3. HPLC analysis of *Caenorhabditis elegans* organic osmolyte content. *A*: HPLC chromatograms of a perchloric acid extract of worms acclimated to 200 mM NaCl for 2 wk. Both chromatograms were derived from the same extract. The chromatogram on the *right*, however, was diluted 1:1 with 2.5 mM glycerol stock. The peak marked with an asterisk remains largely unchanged, whereas all other peaks are reduced by ~50%. This result suggests that the marked peak is glycerol. The presence of glycerol in the samples was confirmed by enzyme assay (see RESULTS). *B*: glycerol levels measured by HPLC analysis in worms acclimated to 200 or 300 mM NaCl for 2–4 wk. Values are means ± SE ($n = 4$ or 5). *$P < 0.001$ compared with control; **$P < 0.001$ compared with control and compared with 200 mM NaCl. *C*: time course of glycerol accumulation in worms exposed to 200 mM NaCl. Values are means ± SE ($n = 4$–9).

to modest dehydration (33, 34). These observations suggested that the unidentified solute might be glycerol, which is expected to elute close to taurine. To test this hypothesis, we added 50 μl of a 2.5 mM glycerol stock to 50 μl of the worm extract shown in Fig. 3*A*, *left*. Data in Fig. 3*A*, *right*, demonstrate that the area of the peak marked with an asterisk remains largely unchanged while other solutes in the sample are diluted ~50%. These results suggest that the solute accumulating in worms during hypertonic stress is glycerol.

To confirm the presence of glycerol in the samples, we performed glycerol measurements with a commercially available enzyme assay kit. In synchronized young adult worms descended from animals exposed for 2–4 wk to 200 or 300 mM NaCl, mean ± SE glycerol levels determined by enzyme assay were 811 ± 142 ($n = 3$) and 1,305 ± 54 ($n = 3$) nmol/mg protein, respectively. These values were not significantly ($P > 0.3$) different from those measured with HPLC shown in Fig. 3*B*. Glycerol levels increased ~17- and ~33-fold, respectively, from a control level of 38 nmol/mg protein in worms acclimated to 200 or 300 mM NaCl agar. We conclude that *C. elegans* accumulates large quantities of the organic osmolyte glycerol when exposed to hypertonic stress.

Figure 3*C* shows the time course of glycerol accumulation in synchronized young adult worms exposed to 200 mM NaCl agar for 3–48 h. Glycerol levels increased approximately threefold ($P < 0.05$) within 3 h after exposure to hypertonicity and peaked after 24 h. Glycerol levels in synchronized young adults acutely exposed to 200 mM NaCl for 24 h (Fig. 3*C*) were not significantly different ($P > 0.6$) from levels observed in synchronized young adults descended from animals chronically exposed to 200 mM NaCl for 4 wk (Fig. 3*B*).

Mechanism of glycerol accumulation. Organic osmolyte accumulation can be mediated either by transport from the extracellular medium or by synthesis from metabolic precursors (6). The experiments shown in Fig. 3 were performed with worms cultured on lawns of *E. coli*. Under hypertonic stress, *E. coli* accumulates the organic osmolytes proline and glycine betaine (41). However, it is conceivable that even small quantities of glycerol produced by the bacteria could be transported across the intestine of *C. elegans* and accumulated intracellularly during hypertonic stress. To test this possibility, late L4 larvae and young adults were transferred to standard M9 buffer for 2 h to evacuate bacteria from the gut. After washing, the worms were placed on 200 mM NaCl agar in the absence of bacteria for 6 h and then prepared for glycerol analysis. Mean ± SE glycerol content was 223 ± 7 nmol/mg protein ($n = 3$). This value was not significantly ($P > 0.4$) different from that observed in worms exposed for 6 h to 200 mM NaCl in the presence of bacteria (Fig. 3*C*). These results demonstrate

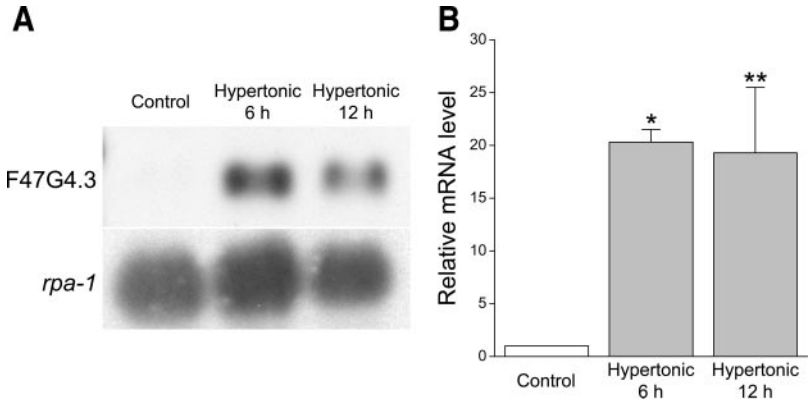

Fig. 4. Effect of hypertonicity on mRNA levels of the *C. elegans* GPD F47G4.3. *A*: Northern blots showing mRNA levels of F47G4.3 and the loading control probe *rpa-1* in control worms and worms acclimated to 175 mM NaCl agar for 6 and 12 h. *B*: relative changes in F47G4.3 mRNA levels in worms acclimated to 175 mM NaCl agar for 6 and 12 h. Values are means ± SD (n = 2–4). Relative mRNA levels of worms exposed to 175 mM NaCl are significantly different from 1. *$P < 0.03$; **$P < 0.01$.

that glycerol is not transported from an exogenous source and instead is synthesized from metabolic precursors.

Very little is known about metabolism in *C. elegans*, and the biochemical pathways that mediate glycerol synthesis have not been identified. Therefore, to further examine the role of synthesis in glycerol accumulation, we quantified mRNA levels of GPD. In yeast, GPD is rate limiting for glycerol synthesis and expression of GPD1 is upregulated by hypertonic stress (1, 38).

A search of the *C. elegans* genome sequence revealed two yeast GPD1 homologs, K11H3.1 and F47G4.3. We performed Northern blot analysis to determine whether either of these genes was transcriptionally regulated by hypertonic stress. As shown in Fig. 4, F47G4.3 mRNA levels were elevated ~20-fold when worms were exposed to 175 mM NaCl for 6 and 12 h. No significant ($P > 0.1$; $n = 3$ experiments) changes in K11H3.1 mRNA levels were detected over the same time course (data not shown). These results suggest strongly that hypertonicity-induced increases in glycerol synthesis are mediated at least in part by increased expression and activity of the GPD1 homolog F47G4.3.

Glycerol loss. If glycerol is required for adaptation to hypertonic stress, its concentration should decrease rapidly when extracellular osmolality is reduced. Worms were acclimated to 200 mM NaCl agar for >4 wk and then transferred back to normal growth medium containing 21 mM NaCl. As shown in Fig. 5*A*, glycerol levels fell rapidly and returned to control levels within 12 h.

In most cells, organic osmolyte concentration is reduced by rapid efflux to the extracellular medium (3, 17, 30, 43). To determine whether efflux contributed to the reduction of glycerol levels shown in Fig. 5*A*, we acclimated worms to 200 mM NaCl agar for >4 wk and then transferred them to 520 or 190 mosmol/kgH_2O M9 buffer for 2 h. Worms exposed to hypertonic M9 excreted ~30 nmol glycerol/mg total worm protein (Fig. 5*B*). Glycerol excretion increased approximately fivefold when high-NaCl-acclimated worms were exposed to 190 mosmol/kgH_2O M9 buffer. As shown in Fig. 2*B*, high-NaCl-acclimated worms swell rapidly when returned to normal growth agar containing 21 mM NaCl. Swelling likely triggers efflux of intracellular glycerol into the pseudocoelomic fluid. Excretion of extracellular glycerol into the external environment may be mediated by the animal's excretory system and/or the intestinal epithelium (42).

DISCUSSION

The similarities in the responses of *C. elegans* and yeast to osmotic stress are noteworthy. Both of these eukaryotes accumulate glycerol via synthesis from metabolic precursors when exposed to hypertonicity (Fig. 3; Ref. 1). Increased expression of GPD1 in yeast mediates glycerol accumulation and is essential for growth and survival during hypertonic stress (1). Transcriptional upregulation of the GPD1 homolog F47G4.3 suggests that this enzyme plays a central role in increasing glycerol levels in nematodes (Fig. 4).

In response to a reduction in extracellular osmolality, glycerol levels in yeast are rapidly reduced by efflux to the extracellular medium. Efflux is mediated by the aquaglyceroporin homolog Fps1 (24). Glycerol levels fall rapidly when 200

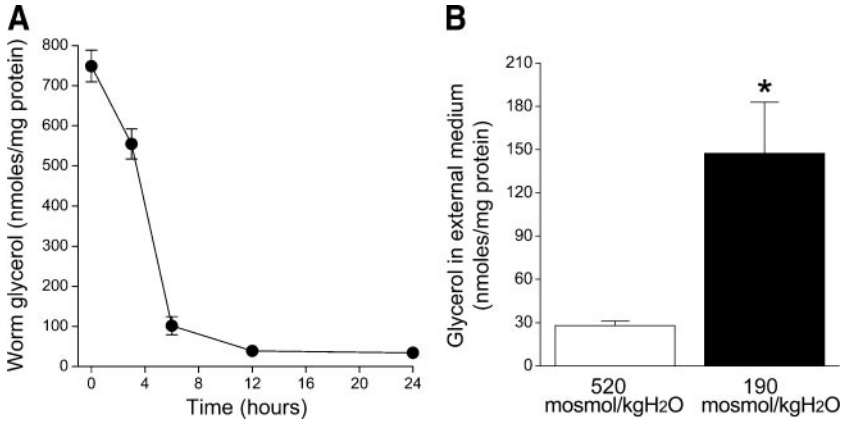

Fig. 5. Glycerol levels and glycerol excretion in worms recovering from hypertonic stress. *A*: time course of glycerol loss in worms acclimated to 200 mM NaCl for >4 wk and then transferred back 21 mM NaCl growth agar. Values are means ± SE (n = 3 or 4). *B*: excretion of glycerol into the external medium. Worms were acclimated to 200 mM NaCl for >4 wk and then transferred to 520 or 190 mosmol/kgH_2O M9 buffer for 2 h. Values are means ± SE (n = 4–7). *$P < 0.02$ compared with 520 mosmol/kgH_2O M9 buffer.

mM NaCl-acclimated worms are exposed to low-NaCl agar (Fig. 5*A*). Reduction of glycerol levels is mediated at least in part by increased glycerol excretion (Fig. 5*B*). Interestingly, we have observed by microarray and Northern blot analysis that a nematode aquaglyceroporin homolog is transcriptionally upregulated three- to sixfold by hypertonic stress (unpublished observations). It is conceivable that this channel mediates glycerol efflux in a manner analogous to that of Fps1 in yeast.

Although there are similarities between yeast and worm osmoregulation, there are also differences. The most interesting and obvious difference is in the upstream signaling elements that regulate glycerol accumulation. As noted above, osmotic stress-induced glycerol synthesis in yeast is controlled by the HOG MAPK cascade (29). HOG signaling consists of two redundant upstream branches that are controlled by the structurally unrelated membrane-spanning proteins Sho1 and Sln1 (14, 41). Recently, a third putative membrane protein, Msb2, was shown to function in parallel with the Sho1 branch (28).

Sln1 is a so-called hybrid histidine kinase that contains both a kinase and a receiver domain. The kinase domain autophosphorylates a histidine residue, and the phosphoryl group is then subsequently transferred to a receiver domain aspartyl residue (40). Sln1 is thought to function as an osmosensor (37), and hypertonic stress inhibits the Sln1 kinase, resulting in activation of the HOG signaling pathway (32).

ATHK1 and Cre1 are hybrid histidine kinases identified in *Arabidopsis thaliana* (15, 44). Both kinases complement yeast Sln1-deletion mutants (37, 44). Recent studies (37) suggest that Sln1 and Cre1 detect changes in turgor pressure, possibly through interaction of the extracellular domains of the proteins with the cell wall.

The signaling pathways that control hypertonicity-induced glycerol accumulation in *C. elegans* are unknown. Homologs of the yeast HOG signaling pathway MAPKs are present in nematodes (4, 19). However, *C. elegans* and other animals do not possess Sho1, Sln1, and Msb2 homologs. Unlike plant cells and yeast, animal cells lack cell walls and are unable to generate significant hydrostatic or turgor pressure. Therefore, an important step in the evolution of animal cells must have involved the development of other mechanisms to sense osmotic stress. Studies in mammalian cells suggest that a hypertonicity-induced increase in intracellular ionic strength is the signal that activates organic osmolyte accumulation mechanisms (27).

C. elegans provides unique experimental opportunities for identifying animal cell volume sensors and osmoregulatory signaling pathways. Molecular description of the HOG pathway was due largely to the molecular tractability of yeast and the ability to perform mutagenesis and forward genetic analysis. *C. elegans* is well suited for similar studies; the animals have a short life cycle, reproduce by self-fertilization or mating with males, and produce large numbers of offspring (42). We recently initiated mutagenesis studies to identify genes involved in cellular osmoregulation and have isolated >30 mutants with an altered ability to survive hypertonic stress. In addition, mutagenesis screens of other biological processes have identified genes that may participate in osmotic homeostasis (18, 23). *C. elegans* and possibly *Drosophila* represent the only animal models in which forward genetic analysis can be used to define the molecular basis of cellular osmoregulation.

In conclusion, we have described for the first time the osmoregulatory response during acclimation to and recovery from hypertonic stress in the nematode *C. elegans*. These studies provide an essential foundation for identifying the genes and genetic pathways required for regulating cellular solute and water balance. The ability to sense and reverse changes in cell volume or turgor pressure is common to all organisms. Osmoregulatory mechanism almost certainly arose early in cellular evolution. Given the likelihood that such mechanisms are conserved across species, genetic analysis of osmoregulation in *C. elegans* will likely provide important insights into molecular mechanisms of osmotic homeostasis in animal cells in general.

GRANTS

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