Copyright © 2006 by the Genetics Society of America
DOI: 10.1534/genetics.106.059089

Identification of a Novel Gene Family Involved in Osmotic Stress Response in *Caenorhabditis elegans*

Jeanna M. Wheeler[1] and James H. Thomas[2]

Department of Genome Sciences, University of Washington, Seattle, Washington 98195

Manuscript received April 6, 2006
Accepted for publication August 21, 2006

ABSTRACT

Organisms exposed to the damaging effects of high osmolarity accumulate solutes to increase cytoplasmic osmolarity. Yeast accumulates glycerol in response to osmotic stress, activated primarily by MAP kinase Hog1 signaling. A pathway regulated by protein kinase C (PKC1) also responds to changes in osmolarity and cell wall integrity. *C. elegans* accumulates glycerol when exposed to high osmolarity, but the molecular pathways responsible for this are not well understood. We report the identification of two genes, *osm-7* and *osm-11*, which are related members of a novel gene family. Mutations in either gene lead to high internal levels of glycerol and cause an osmotic resistance phenotype (Osr). These mutants also have an altered defecation rhythm (Dec). Mutations in cuticle collagen genes *dpy-2*, *dpy-7*, and *dpy-10* cause a similar Osr Dec phenotype. *osm-7* is expressed in the hypodermis and may be secreted. We hypothesize that *osm-7* and *osm-11* interact with the cuticle, and disruption of the cuticle causes activation of signaling pathways that increase glycerol production. The phenotypes of *osm-7* are not suppressed by mutations in MAP kinase or PKC pathways, suggesting that *C. elegans* uses signaling pathways different from yeast to mount a response to osmotic stress.

ALL living organisms have mechanisms that allow them to cope with changing environmental conditions such as temperature, food availability, the presence of toxic compounds, and osmolarity. When an organism or tissue is exposed to high osmolarity, intracellular water rapidly diffuses across cell membranes, causing a decrease in volume and increased concentration of cellular contents. These changes lead to mechanical stress, disruption of the activity of proteins and DNA, and eventually shutdown of cellular function (Garner and Burg 1994). To avoid this outcome, cells accumulate physiologically compatible solutes that increase intracellular osmolarity without a concomitant disruption of ionic bonds due to increased salt concentration. For instance, mammalian kidney cells must endure large variation in solute concentrations, depending on the hydration state of the animal. In response, these cells increase production or transport of compatible solutes including sorbitol, inositol, betaine, and taurine (Burg *et al.* 1997).

Adaptation to osmotic stress has been studied extensively in yeast (Hohmann 2002), where glycerol is used as a compatible solute. The MAP kinase Hog1 is required for this response (Brewster *et al.* 1993) and indirectly activates Gpd1, a glycerol-3-phosphate dehydrogenase shown to be rate limiting for glycerol accumulation (Albertyn *et al.* 1994; Remize *et al.* 2001). Mutants in the Hog1 pathway are sensitive to high osmolarity, but this sensitivity is alleviated by growth at high temperature (Siderius *et al.* 2000). The most likely explanation for this alleviation is the activation of a second pathway involving protein kinase C (Pkc1), which responds to high temperature, cell wall damage, and osmotic stress (Gustin *et al.* 1998). In other systems, PKC is known to be activated by diacylglycerol produced by phospholipase C (PLC). While direct evidence of this interaction is lacking in yeast, it has been shown that yeast PLC (Plc1) is required for increased glycerol synthesis in response to osmotic stress (Lin *et al.* 2002).

While the response to high osmolarity in yeast is well understood, the response of the nematode *Caenorhabditis elegans* has only recently been examined and remains poorly understood. In the natural soil environment of *C. elegans*, osmolarity probably varies widely and changes rapidly. Because *C. elegans* has a high surface-to-volume ratio and water passes freely through its cuticle, worms are particularly vulnerable to osmotic stress. The primary mechanism that *C. elegans* uses for protection from osmotic stress is locomotion away from an area of hyperosmolarity (Culotti and Russell 1978). Nematode locomotion is achieved by alternating contractions of body wall muscles that are connected to the cuticle. Internal hydrostatic pressure on the cuticle gives the animal rigidity, against which locomotory muscles work. Thus, *C. elegans* has a hydrostatic skeleton, and locomotion is dependent on turgor pressure to maintain the rigidity of the animal. Hyperosmotic conditions lead to loss of internal pressure by water efflux, collapse of the

[1]*Present address:* Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR 97239.

[2]*Corresponding author:* 1705 NE Pacific St., Box 357730, Seattle, WA 98195-7730. E-mail: jht@gs.washington.edu

hydrostatic skeleton, and rapid paralysis of *C. elegans.* To avoid death induced by hyperosmotic shock, *C. elegans* has evolved mechanisms that maintain turgor pressure and allow for continued locomotion.

Similar to yeast, *C. elegans* increases glycerol synthesis to attenuate the damaging effects of high osmolarity. Worms that have been exposed to high levels of environmental NaCl contain high internal levels of glycerol and show increased expression of the glycerol-3-phosphate dehydrogenase F47G4.3 (Lamitina *et al.* 2004). However, it has not been determined whether MAP kinase, PKC, or any other previously identified pathways are involved in this response. Mutations in one gene, *osr-1*, have previously been shown to cause resistance to high osmolarity (Solomon *et al.* 2004). OSR-1 is a novel protein expressed in the hypodermis and likely secreted. *osr-1* mutants have high basal levels of glycerol, even under normal growth conditions, and this is thought to be the reason for their osmotic resistance.

We describe here mutations in two additional genes, *osm-7* and *osm-11*, that cause resistance to high osmolarity. Like *osr-1*, these mutations also lead to high basal levels of glycerol. In addition, we show that the defecation rhythm of Osr worms is altered in a manner similar to that of worms exposed to chronic osmotic stress. Both *osm-7* and *osm-11* are members of a novel gene family in *C. elegans* and are likely to encode secreted proteins. We show that signaling components of the MAP kinase and PKC pathways are not required for the phenotypic changes observed in *osm-7* mutants. Finally, we discuss the possibility that Osr genes may be important for cuticle integrity and that certain defects in the cuticle lead to activation of the same signaling pathway that responds to osmotic stress.

MATERIALS AND METHODS

***C. elegans* strains:** Strains used in this study were N2 Bristol wild type, JT89 *osm-7(sa89)*, MT3564 *osm-7(n1515)*, MT3643 *osm-11(n1604)*, RB1032 *osr-1(ok959)*, BE93 *dpy-2(e8)*, CB128 *dpy-10(e128)*, CB88 *dpy-7(e88)*, CB61 *dpy-5(e61)*, CB184 *dpy-13(e184sd)*, CB164 *dpy-17(e164)*, CB364 *dpy-18(e364am)*, CB266 *unc-43(e266)*, MT2605 *unc-43(n1186 n498sd)*, CX4998 *nsy-1(ky397)*, KU4 *sek-1(km4)*, JT28 *egl-8(sa28)*, MJ500 *tpa-1(k501)*, RB781 *pkc-1(ok563)*, VC127 *pkc-2(ok328)*, JT734 *goa-1(sa734)*, PS2444 *dpy-20(e1282ts); egl-30(syIs36)*, JT11445 *osm-7(sa89); saEx687*, JT11446 *osm-7(sa89); saEx688*, GE2175 *unc-32(e189) tDf6/qC1 dpy-19(e1259) glp-1(q339); him-3(e1147)*, GE2204 *unc-32(e189) tDf10/qC1 dpy-19(e1259) glp-1(q339); him-3(e1147)*, and MT1642 *lin-15(n765ts)*. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources.

Mapping and transgenic rescue: *osm-7(sa89)* was previously mapped to the right arm of chromosome III by standard mapping crosses (Iwasaki *et al.* 1995). Deletion mapping was carried out by crossing *osm-7(sa89)* to GE2175 and GE2204, strains carrying the deletions *tDf6* and *tDf10*, respectively. We observed that *sa89/tDf6* and *sa89/tDf10* animals are Dec, indicating that *osm-7* is located in a small region of chromosome III to the right of *unc-64*. Cosmids in this region were obtained from the Sanger Institute (Cambridge, UK) and transgenic lines were made using standard methods. The exon structure of T05D4.4 was confirmed by sequencing of cDNA clones (yk723f1 and yk727g8) provided by Yuji Kohara (National Institute of Genetics, Mishima, Japan). We confirmed that *sa89* and *n1515* are alleles of the same gene by a complementation test. *n1515*/+ males were crossed to *sa89; dpy-9(e12)* hermaphrodites, and half of the resulting cross progeny were Dec.

Gene structure: The predicted exon structure of genes K02F3.7 and ZK507.4 lacked experimental support in the WS143 release of WormBase and their predicted proteins had regions of poor alignment with experimentally confirmed members of the osmotic resistance (OSR)-domain family. *tblastn* searches and manual inspection were used to modify their predictions as follows. K02F3.7 had one internal exon added and an intron was added inside the first exon. ZK507.4 had its last exon shortened and a new terminal exon added. All changes used plausible splice sites and resulted in dramatically improved alignments with other family members. All changes have been reported to WormBase.

Behavioral assays: For all defecation assays, worms were raised and scored at 20°. For each strain, 10 L4 animals were picked to a fresh NGM plate seeded with OP50 and assayed as adults the following day for 10 min each. Statistical significance was determined using the Mann-Whitney *U*-test.

High-salt plates were poured 3 days before use and seeded with OP50 1 day before use to minimize variation in salt concentration due to evaporation. For acute stress assays, worms ($N \geq 70$) raised on standard 50-mm NGM plates were picked onto high-salt plates and assayed by touch 10 min later. Response to touch was counted as any attempt at locomotion, even weak movements. For chronic osmotic stress assays, animals ($N \geq 80$) were placed on high-salt plates and removed after 24 hr using M9 buffer containing 300 mm NaCl. The animals were allowed to recover for 24 hr before viability was assayed. Statistical significance was determined using Fisher's exact test.

To measure resistance to oxidative stress, adult worms were picked onto NGM plates containing 50 mm paraquat and allowed to lay eggs ($N \geq 100$). Viability was calculated as the number of eggs hatched after 24 hr. For heat stress assays, adult worms ($N \geq 80$) were shifted to 35°, and the same individuals were assayed for response to touch every 2 hr. Statistical significance was determined using Fisher's exact test.

Cuticle disintegration was measured as previously described by Watts *et al.* (2003). Adult nematodes ($N = 15$) were placed in 200 μl of alkaline hypochlorite solution (1% sodium hypochlorite, 0.25 m NaOH) in a 96-well culture plate and observed under a dissecting microscope until the first break in the cuticle occurred. Statistical significance was determined using Student's *t*-test.

RNAi experiments: Genomic fragments were amplified from T05D4 using GenePairs primer sequences (Research Genetics, Birmingham, AL), cloned into pCR TOPO vector (Invitrogen, San Diego), and then moved into the L4440 vector and transformed into the HT115 bacterial strain as described (Fraser *et al.* 2000). The bacterial strain used for knockdown of F11C7.5 was obtained from the Ahringer RNAi library (MRC Geneservice). Growth plates used for RNAi were prepared as previously described (Fraser *et al.* 2000). L4 animals were placed on dsRNA-producing bacteria and raised at 15° for 72 hr and then transferred to fresh plates at 20°. Progeny from the second set of plates were assayed 4 days later for the Dec or Osr phenotype.

Determination of glycerol content: Each assay was done in triplicate on at least two separate days. Statistical significance was determined using Student's *t*-test. Well-fed worms were

TABLE 1

Phenotypes of Osr mutants

Strain	500 mM NaCl (%)	800 mM NaCl (%)	1 M NaCl (%)	800 mM sucrose (%)	Glycerol content (ng/mg protein ± SD)
N2	3	0	0	2	3.2 ± 0.7
osm-7	100	100	40	100	159.8 ± 28.9
osm-11	100	100	43	100	141.8 ± 6.6
osr-1	97	27	0	98	42.4 ± 4.9
dpy-2	96	7	ND	ND	ND
dpy-10	97	6	ND	ND	42.5 ± 15.9
dpy-7	100	10	ND	ND	39.1 ± 9.7
dpy-5	6	0	ND	ND	ND
dpy-13	10	0	ND	ND	ND
dpy-17	4	0	ND	ND	ND
dpy-18	3	0	ND	ND	ND

Resistance to acute osmotic stress was measured as the percentage of animals ($N = 70$) responding to touch after a 10-min exposure to NaCl or sucrose. Glycerol content is shown as the mean ± standard deviation of six independent assays, normalized to the protein content of each sample. For all assays, all Osr strains are significantly different from N2 ($P < 0.0001$).

rinsed off plates with M9, spun down, and then rinsed with fresh M9 and resuspended in 600 μl M9. Worms were sonicated 2 × 30 sec and disruption of the cuticle was confirmed using a dissecting microscope. Insoluble material was removed by centrifugation for 5 min at maximum speed. The supernatant from each sample was divided, 50 μl were used for measurement of protein content with a BCA protein assay kit (Pierce, Rockford, IL) and 200 μl were used for measurement of glycerol content with an enzymatic assay (R-Biopharm). In both cases, the assays were performed as described in the product instructions. Prior to measurement of glycerol content, a Carrez clarification was performed to remove protein: the sample was brought up to 600 μl with water, and then 50 μl each of 85 mM ferrocyanide and 250 mM zinc sulfate were added. The pH was adjusted to 8.0 using 1 M NaOH, and the final volume was brought up to 1 ml. Proteins were pelleted by centrifugation for 15 min at maximum speed.

GFP expression: A PCR fragment containing 5 kb of *osm-7* genomic sequence was amplified and inserted into *Bam*HI and *Sph*I sites in the Fire vector p95.67 with GFP fused in frame to the second exon. This construct was injected into *lin-15(n765ts)* worms at 100 ng/μl, along with a *lin-15(+)* marker construct at 60 ng/μl.

RESULTS

Osr phenotype of *osm-7* and *osm-11*: *osm-7(n1515)* and *osm-11(n1604)* were originally identified in a screen for mutants with defective osmotic avoidance (Osm) (J. Thomas, unpublished results). It was later noted that these mutants are also resistant to high osmolarity (Osr) (K. Mase, Y. Ohshima and M. Koga, personal communication). *dec-2(sa89)* was identified in a separate screen for mutants with abnormal defecation cycle period (Dec) (Iwasaki *et al.* 1995). *osm-7(n1515)* and *dec-2(sa89)* are alleles of the same gene; thus we refer to this gene as *osm-7* for the remainder of the article.

Upon exposure to 500 mM NaCl, wild-type *C. elegans* ceases egg laying and becomes notably deflated. Within minutes, the loss of water by osmosis leads to complete paralysis. For a more detailed description of the wild-type response to osmotic stress, refer to Lamitina *et al.* (2004). We measured the Osr phenotype as locomotion in response to touch after a 10-min exposure to high salt. Table 1 shows the Osr phenotype of several mutants. In sharp contrast to N2, *osm-7* and *osm-11* worms moved normally and laid eggs at 500 mM NaCl. *osr-1* animals were somewhat resistant and continued to move, but appeared slightly deflated and sluggish. At 800 mM NaCl, the difference between *osr-1* and *osm-7/osm-11* was more pronounced. Under these extreme conditions, a majority of *osr-1* animals were paralyzed, yet *osm-7* and *osm-11* animals appeared completely unaffected. As previously reported for *osr-1* (Solomon *et al.* 2004), *osm-7* and *osm-11* are also resistant to high concentrations of sucrose (Table 1), but not to heat or oxidative stress (Table 2), indicating that these mutants are specifically resistant to osmotic stress.

Effect of cuticle defects on osmotic resistance: The cuticle collagen mutants *dpy-2* and *dpy-10* were previously reported to exhibit the Osr phenotype (Solomon *et al.* 2004) and thus were included in the present study. In our assay, *dpy-10* mutants were resistant to 500 mM NaCl, but like *osr-1* were less tolerant of 800 mM NaCl. *dpy-2* and *dpy-10* mutations have been shown to affect the localization of DPY-7, another cuticle collagen (McMahon *et al.* 2003). *dpy-7* mutants also displayed resistance to 500 mM NaCl, whereas *dpy-5*, *dpy-13*, *dpy-17*, and *dpy-18* mutants did not (Table 1). This indicates that not all types of cuticle defect can cause osmotic resistance.

We have considered the possibility that *osm-7* and *osm-11* mutant animals might have structural cuticle defects. Two pieces of evidence suggest that this is not the case. First, these mutants do not exhibit gross morphological defects at any stage of development. They are somewhat scrawny and slow growing, but do not appear shorter than normal (Dumpy phenotype). Second, *osm-7* and

TABLE 2

Response of Osr mutants to other types of stress

Strain	35° heat shock (%) 4 hr	6 hr	8 hr	Oxidative stress: % eggs hatched	Cuticle disintegration: Avg. sec
N2	98	74	12	67	362 ± 77
osm-7	98	78	14	66	405 ± 111
osm-11	92	72	16	64	376 ± 104
daf-2	98	90*	52**	96**	—
dpy-10	—	—	—	—	181 ± 17**

Resistance to heat shock was measured as the percentage of animals ($N = 80$) responding to touch after 4, 6, or 8 hr of incubation at 35°. Resistance to oxidative stress was measured as the percentage of eggs ($N \geq 100$) laid on plates containing 50 mM paraquat that hatched after 24 hr. Cuticle disintegration is the average time to the first major cuticle break when worms are exposed to alkaline hypochlorite solution ($N = 15$). *$P < 0.01$ compared to N2. **$P < 0.0001$ compared to N2.

osm-11 animals have a wild-type level of sensitivity to a cuticle-disrupting treatment (Table 2). This indicates that not all Osr genes are required for the structural integrity of the *C. elegans* cuticle.

Relationship between osmotic resistance and defecation behavior: Since *osm-7(sa89)* was known to alter the defecation cycle period, we examined the defecation phenotype of the other Osr mutants. Under normal laboratory growth conditions, wild-type worms execute the defecation motor program every 45–50 sec. This behavior occurs with a strikingly regular periodicity, but the length of the period can be altered by variables such as food concentration and application of light touch. As shown in Figure 1, all of the known Osr mutants have an altered defecation cycle period.

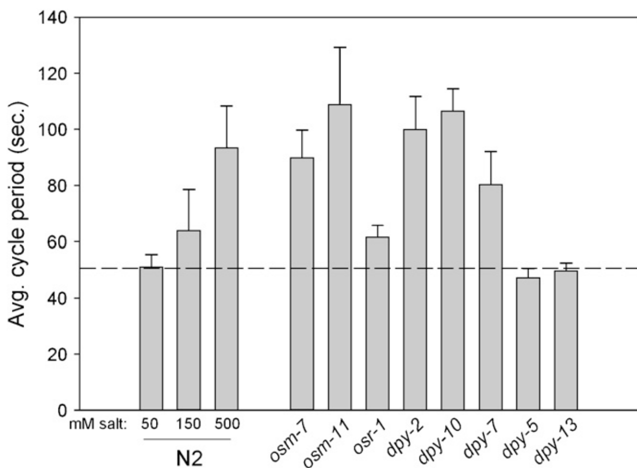

Figure 1.—Correlation between Dec and Osr phenotypes. N2 worms have altered cycle periods ($P < 0.0001$) on high-salt plates *vs.* normal NGM agar plates (50 mM NaCl). The defecation cycle period of all Osr mutants is longer than that of wild type ($P < 0.0001$). *dpy-5* and *dpy-13* have normal cycle periods. Average cycle periods are the unweighted average of all measured cycle periods. Error bars show the standard deviation.

The correlation between the Osr and Dec phenotypes suggests that they are mechanistically linked. We hypothesized that exposure to high osmolarity might alter the defecation cycle in wild-type *C. elegans*. Indeed, we found that adult worms exposed to 150 mM NaCl exhibited a modest increase in cycle period (Figure 1). If raised on high-salt plates, wild-type *C. elegans* adapt to osmotic stress by accumulating glycerol (Lamitina *et al.* 2004). In this adapted state, the animals are resistant to subsequent osmotic challenge and can survive exposure to even higher levels of osmotic stress. We observed that worms raised on 200 mM NaCl to permit adaptation and then shifted to 500 mM had cycle periods as long as those of *osm-7* and *osm-11* (Figure 1). Thus, the period of the defecation rhythm is altered in response to osmotic stress, and the length of the cycle is correlated with the osmolarity of the growth medium.

Molecular identification of *osm-7* and *osm-11*: *osm-7* was localized to the right arm of chromosome III using standard mapping crosses, and deletion mapping refined this to a small region near the telomere (Figure 2A). Injection of the cosmid T05D4 rescued the *osm-7* phenotype completely. RNAi of each of the five genes on T05D4 revealed that knockdown of only one gene, T05D4.4, produced a slow-growing, Dec phenotype. In addition, we found a nonsense mutation in the coding sequence of T05D4.4 in the *osm-7(sa89)* strain (Figure 2B). Together these results indicate that *osm-7* encodes the protein T05D4.4.

T05D4.4 is a novel protein with no significant similarity to proteins outside of nematodes or to any previously identified functional domains. However, it does show similarity to several genes in *C. elegans* and *C. briggsae*. Figure 2C shows a partial alignment of OSM-7 with some of its homologs. The greatest similarity is in a region we have named the OSR domain, which is located toward the N terminus of the proteins. The fact that the family is small and the domain is not highly conserved may explain why it was not previously noted. Furthermore, the available gene predictions for K02F3.7

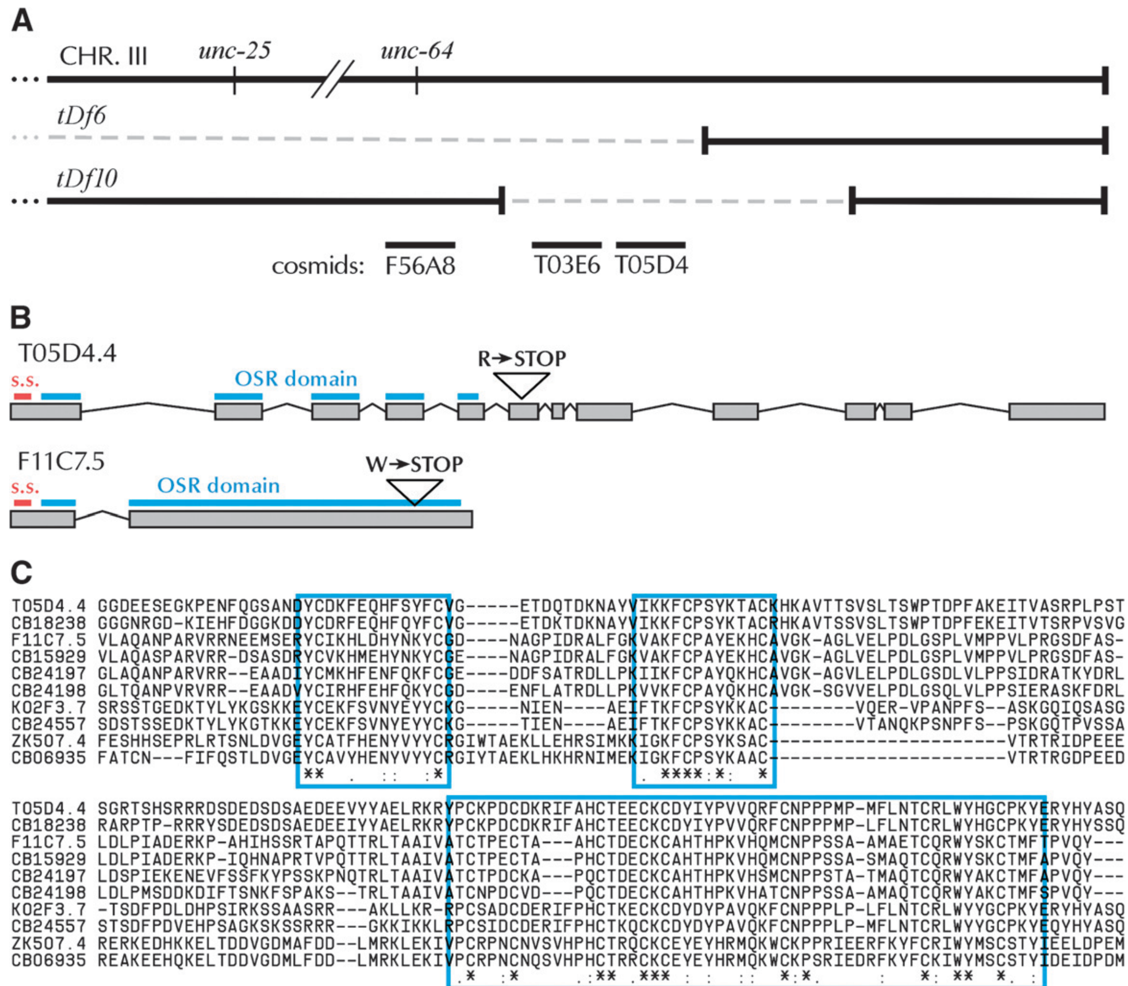

FIGURE 2.—Molecular identification of *osm-7* and *osm-11*. (A) Genomic region of *osm-7*, with deletions and cosmids used for mapping, not to scale. (B) Genomic structure of *osm-7* and *osm-11*, showing locations of signal sequences (s.s., red), OSR domains (blue), and sequenced mutations. (C) CLUSTAL alignment of the *osm-7* OSR domain with its closest relatives from *C. elegans* and *C. briggsae*. Conserved regions of the OSR domain are boxed in blue.

and ZK507.4 were incorrect, decreasing the apparent similarity. Each of the *C. elegans* family members is more closely related to its *C. briggsae* homolog than it is to any other *C. elegans* protein. However, the degree of similarity within these pairs (~75% identical) is lower than average for orthologous gene pairs in these two species. All OSR domain-containing proteins also contain a signal sequence, suggesting that these are secreted proteins. In addition, the position of 12 cysteine residues is conserved in all family members, indicating that they may be extracellular.

osm-11 had been previously mapped to a region containing F11C7.5, which encodes a protein related to *osm-7*. Knockdown of F11C7.5 by RNAi produced a slow-growing, Dec phenotype identical to that of *osm-11(n1604)*. Sequencing of this gene in the *osm-11(n1604)* strain revealed a nonsense mutation in the coding sequence (Figure 2B). Thus, we have shown that at least two members of this family cause the same phenotypes when mutated, suggesting that they have similar but nonredundant functions in the animal.

High glycerol levels in Osr strains: Since *osr-1* animals were previously shown to have high basal levels of glycerol (SOLOMON *et al.* 2004), we hypothesized that other Osr strains might have high glycerol as well. Indeed, all tested Osr strains have elevated levels of glycerol (Table 1), and the elevation in glycerol is strongly correlated with the severity of the Osr phenotype. *osr-1*, *dpy-7*, and *dpy-10*, which had lower glycerol levels than *osm-7* and *osm-11*, are also less resistant to osmotic stress.

An interesting side effect of this high glycerol phenotype is cryoresistance. *C. elegans* strains can be stored at −80° for many years if the worms are suspended in a solution containing 30% glycerol. Approximately 5% of *osm-7* mutant worms were able to survive freezing without any addition of glycerol, whereas wild-type worms never survived this treatment (data not shown).

TABLE 3

Epistasis analysis of Osr mutants

Strain	800 mM NaCl (%)	1 M NaCl (%)	Glycerol content (ng/mg protein ± SD)	Defecation period (sec)
osm-7; osm-11	100	47	ND	103.3 ± 16.0
osm-7; osr-1	100	48	ND	99.7 ± 11.1
osm-7; dpy-2	33*	ND	ND	100.2 ± 6.5
osm-7; dpy-10	42*	ND	78.7 ± 9.7*	96.3 ± 12.0
osm-7; dpy-7	45*	ND	87.9 ± 11.3*	100.5 ± 9.8

Resistance to acute osmotic stress was measured as the percentage of animals ($N = 70$) responding to touch after a 10-min exposure to 800 mM or 1 M NaCl. Glycerol content is shown as the mean ± standard deviation of six independent assays, normalized to the protein content of each sample. Defecation cycle period is the average for 10 animals observed for 10 min each, given in seconds ± standard deviation. *$P < 0.0001$ compared to *osm-7* single mutant.

Genetic interaction of Osr mutants: To characterize the functional relationship between the Osr genes, we constructed double mutants between *osm-7* and five other Osr genes. We expect that if these genes function in separate pathways, their resistance phenotypes would be additive, causing the double mutants to be even more resistant to osmotic stress than the single mutants. *osm-7; osr-1* and *osm-7; osm-11* exhibited resistance phenotypes identical to that of the *osm-7* single mutant at 800 mM NaCl (Table 3). However, because *osm-7* is 100% resistant to 800 mM NaCl, it would not be possible to detect increased resistance. Thus, we also measured the resistance of these strains to a higher level of osmotic stress. At 1 M NaCl, we observed that 40% of *osm-7* animals responded to touch after a 10-min exposure (Table 1), and the responses of *osm-7; osr-1* and *osm-7; osm-11* were similar (Table 3). This result indicates that these three genes may contribute to osmotic resistance by activation of a single pathway.

In contrast, double mutants between *osm-7* and any of the Osr collagen mutants (*dpy-2*, *dpy-7*, and *dpy-10*) had osmotic resistance and glycerol accumulation phenotypes that were intermediate to each single mutant (Table 3). These double mutants were less resistant to 800 mM NaCl than the *osm-7* single mutant, but more resistant than each of the Dpy single mutants. The defecation phenotypes of these double mutants were not significantly altered relative to any of the single mutants, and the Dpy phenotype was not visibly altered by the presence of the *osm-7* mutation. These results are difficult to interpret, since none of the three Dpy mutations represent null alleles. However, this experiment reveals that there is a genetic interaction between *osm-7* and these cuticle collagen genes, and because their phenotypes are not additive it remains possible that they function in a single pathway.

Interaction with MAP kinase and PKC pathways: In both yeast and mammals, a MAP kinase pathway has been shown to mediate the response to osmotic stress by increasing the production of physiologically compatible solutes (Burg *et al.* 1997; Hohmann 2002). In yeast, a parallel pathway involving protein kinase C and phospholipase C has also been shown to respond to osmotic stress (Brewster *et al.* 1993; Lin *et al.* 2002). We hypothesized that if either of these pathways is involved in the nematode response, it might be possible to suppress the phenotypes of *osm-7* using mutants in these pathways. *nsy-1* and *sek-1* are worm homologs of MAPKKK and MAPKK, respectively, and function in both pathogen resistance and neuronal cell fate (Sagasti *et al.* 2001; Kim *et al.* 2002). They have also been implicated in the response to osmotic stress, since mutations in this pathway are reported to partially suppress the Osr phenotype caused by RNAi knockdown of *osr-1* (Solomon *et al.* 2004). *egl-8* encodes phospholipase C, and mutants display defects in locomotion, egg laying, and defecation (Miller *et al.* 1999). *C. elegans* has four protein kinase C homologs, *tpa-1*, *pkc-1*, *pkc-2*, and *pkc-3*, which are homologs of the mammalian PKCδ/θ, PKCε, PKCα/β, and PKCζ, respectively (Tabuse 2002). *pkc-3* has an essential function in embryogenesis and thus was not included in this study. *unc-43* encodes the only CamKII in *C. elegans* and has been shown to function upstream of both *nsy-1* and *egl-8* pathways (Robatzek and Thomas 2000; Sagasti *et al.* 2001).

Double mutants were constructed between *osm-7* and each of the above genes. None of the double mutants were suppressed for their acute or chronic osmotic resistance or for defecation phenotypes relative to *osm-7* animals (Table 4). In addition, all double-mutant strains had glycerol levels as high as those of *osm-7* and *osm-11* (Table 4). We also examined *goa-1(sa734)* and *egl-30(syIs36)* single mutants, which are predicted to cause constitutive activation of the *egl-8* pathway, but neither one exhibited Osr or high glycerol phenotypes (data not shown).

Expression of *osm-7*: For genes known to affect the defecation rhythm, expression is required in the intestine (Take-Uchi *et al.* 1998; Dal Santo *et al.* 1999). In contrast, the only previously studied Osr gene (*osr-1*) is required in the hypodermis for its function (Solomon *et al.* 2004). Thus we wanted to determine where in the

TABLE 4

***osm-7* candidate suppressor screen**

Strain	800 mM NaCl (%)	500 mM NaCl (24 hr) (%)	Glycerol content (ng/mg protein ± SD)	Defecation period (sec)
osm-7	100	100	159.8 ± 28.9	89.8 ± 10.0
N2	0	19	3.2 ± 0.7	51.0 ± 4.5
osm-7; unc-43	100	98	174.8 ± 33.8	96.5 ± 16.7
unc-43	0	15	7.0 ± 0.8	48.9 ± 3.8
osm-7; nsy-1	100	94	180.6 ± 5.9	89.2 ± 9.0
nsy-1	0	20	2.8 ± 0.9	52.5 ± 3.0
osm-7; sek-1	100	97	145.9 ± 6.4	85.3 ± 9.9
sek-1	0	16	4.7 ± 1.2	52.8 ± 5.4
osm-7; egl-8	100	92	166.9 ± 25.8	82.1 ± 18.8
egl-8	0	18	3.3 ± 1.9	49.2 ± 7.3
osm-7; pkc-1	100	100	177.9 ± 36.1	74.2 ± 8.4
pkc-1	0	25	2.5 ± 1.3	53.6 ± 5.7
osm-7; pkc-2	100	100	ND	101.1 ± 15.3
pkc-2	0	28	ND	55.0 ± 4.7
osm-7; tpa-1	100	100	ND	79.9 ± 4.1
tpa-1	0	23	ND	53.6 ± 4.2

Resistance to acute osmotic stress was measured as the percentage of animals ($N = 70$) responding to touch after a 10-min exposure to 800 mM NaCl. Resistance to chronic osmotic stress was measured as the percentage of animals ($N = 80$) surviving after 24 hr exposure to 500 mM NaCl. Glycerol content is shown as the mean ± standard deviation of six independent assays, normalized to the protein content of each sample. Defecation cycle period is the average for 10 animals observed for 10 min each, given in seconds ± standard deviation.

animal *osm-7* is expressed, to distinguish whether its Dec phenotype is a consequence of interaction with other Dec genes in the intestine or is a result of an indirect effect.

We used a GFP fusion that contained ~5 kb of *osm-7* genomic DNA. This fusion included 3 kb of upstream promoter sequence, the first exon, the first intron, and part of the second exon of *osm-7*. Four of four transgenic lines showed expression of GFP in the hypodermis (Figure 3). Expression was not observed in any other tissue, suggesting that *osm-7* expression is not required

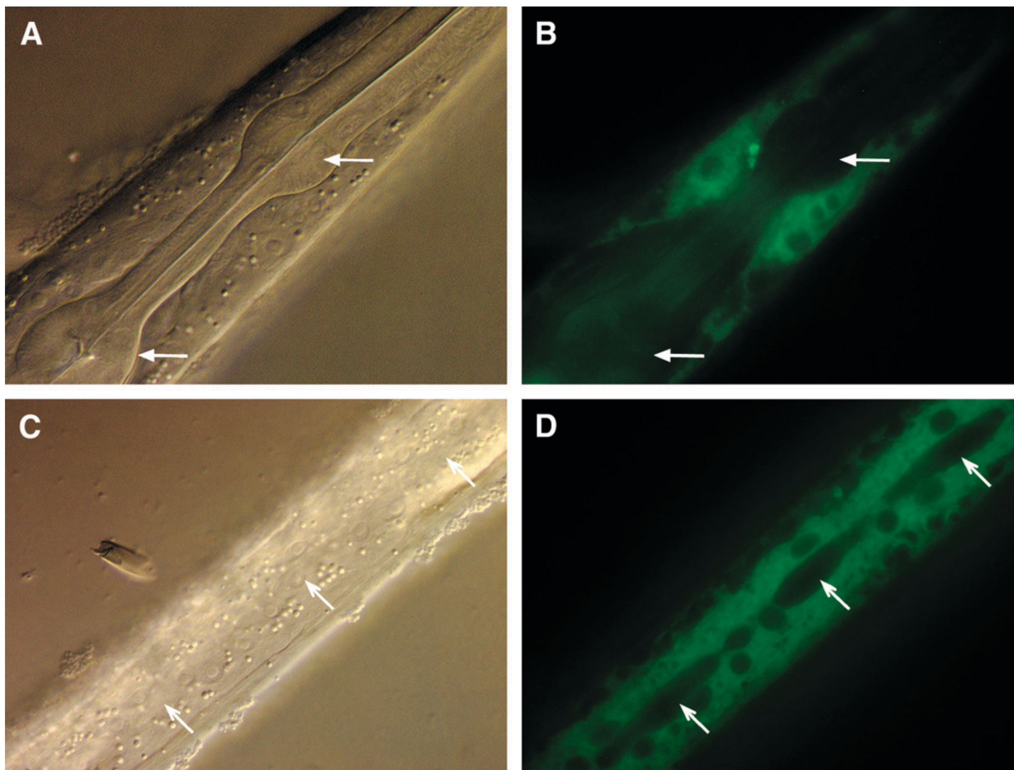

Figure 3.—Expression of *osm-7*::GFP in the hypodermis. Nomarski (A and C) and fluorescence (B and D) images of two adult animals are shown. (A and B) The head region of one animal, with the anterior and posterior bulbs of the pharynx marked by closed arrows. Hypodermal cells expressing GFP are visible along the edges of the animal, surrounding the pharynx (B). (C and D) The midsection of another animal, with GFP-expressing hypodermal cells. Both images are at the top focal plane of the animal, so that the hypodermis is in focus but the intestine and other internal structures are not visible. The seam cells, which are not readily apparent in C, become visible as non-GFP cells along the center of the animal (open arrows) in D.

in the intestine. However, since OSM-7 appears to be a secreted protein, this result does not reveal the site of action of the protein. OSM-7 function may not be required in the hypodermal cells themselves, but rather at an extracellular location such as the cuticle or pseudocoelomic space.

DISCUSSION

We have described the identification of a novel gene family in *C. elegans* and phenotypic analysis of mutations in two members of this family. There are no homologs to this family outside of nematodes, and the family members themselves show significant similarity to each other only within a region we have named the OSR domain. Members of this family show ~75% protein sequence identity to their respective *C. briggsae* orthologs, which is below the median value for ortholog pairs in these two species (Stein *et al.* 2003). This may indicate that this family is evolving more rapidly than average for *C. elegans* proteins. The exact function of the OSR domain is not known, but it contains a large number of highly conserved cysteine residues that are often important for protein stability in an extracellular environment. All family members also contain a signal sequence, which indicates targeting to a vesicular pathway and is also suggestive of an extracellular function.

We have shown that at least two members of this family have similar functions *in vivo*, on the basis of their nearly identical mutant phenotypes. Both *osm-7(sa89)* and *osm-11(n1604)* cause a long defecation cycle, resistance to high osmolarity, and high internal glycerol levels. The mutants *osr-1(ok959)*, *dpy-2(e8)*, *dpy-7(e88)*, and *dpy-10(e128)* also display similar phenotypes (Solomon *et al.* 2004 and our results). Previous work has shown that *C. elegans* accumulates glycerol in response to osmotic stress (Lamitina *et al.* 2004). We have shown that similar osmotic conditions also cause a long defecation cycle period in wild-type worms. While it seems clear that high internal glycerol is the cause of the osmotic resistance phenotype in our mutants, the relationship between these phenotypes and the defecation phenotype is not clear. The phenotypes of the *dpy-7* and *dpy-10* mutants (intermediate glycerol levels and very long cycle period) show that glycerol levels are not directly correlated with defecation cycle period. Thus, it is unlikely that glycerol concentration is the direct cause of altered defecation behavior in the Osr mutants. It is possible that the same signaling molecules that act downstream of *osm-7* to trigger glycerol accumulation also interact with the defecation clock (Dal Santo *et al.* 1999) in the intestine. Alternatively, the response to high osmolarity may trigger a more general stress response in parallel to the specific glycerol response, and this general stress response may be responsible for alteration of the defecation cycle. Consistent with this possibility, we have observed that RNAi knockdown of many genes, including several ribosomal proteins, causes a Dec phenotype (J. M. Wheeler, unpublished results), suggesting that the defecation clock is affected by general metabolic stress.

dpy-2, *dpy-7*, and *dpy-10* encode collagens that are required for proper formation of circumferential furrows, called annuli, on the surface of the *C. elegans* cuticle (McMahon *et al.* 2003). The observation that these mutants also display Osr and Dec phenotypes is intriguing, since it suggests that defects in the cuticle can trigger the same response as osmotic stress. Furthermore, other cuticle defects that cause a Dpy phenotype, such as *dpy-5*, *dpy-13*, *dpy-17*, and *dpy-18*, do not cause Osr or Dec phenotypes. DPY-5 and DPY-13 are cuticle collagens that localize to wide interfurrow bands and are not required for the localization of DPY-7 to the annular furrows (McMahon *et al.* 2003). DPY-17 is a cuticle collagen required for the formation of a structure in embryonic annular ridges (Novelli *et al.* 2006), and DPY-18 is a collagen-modifying enzyme (Winter and Page 2000). This suggests that the DPY-2/-7/-10 annular furrow substructure is specifically involved in osmotic stress response. We propose a model in which the circumferential bands of proteins that form the annuli act as stretch sensors that monitor the turgor pressure of the cuticle. This may be analogous to the case in yeast, where cell wall defects activate the PKC1 pathway (de Nobel *et al.* 2000) and overexpression of cell wall biosynthesis genes alleviates the growth defect of Hog1 MAPK pathway mutants (Alonso-Monge *et al.* 2001). In addition, the yeast genes Wsc1 and Mid2 function upstream of Pkc1 as sensors of osmotic stress at the cell surface (Hohmann 2002). These parallels raise the possibility that *osm-7* and *osm-11* are associated with the cuticle and are responsible for transducing the stretch signal from the annuli. This hypothesis is supported by our evidence for genetic interactions between *osm-7* and three cuticle collagen genes. This would also be consistent with our finding that *osm-7* is expressed in the hypodermis and with the evidence that it is a secreted protein but does not appear to play a structural role. *osr-1* was also shown to be expressed in the hypodermis and to contain a signal sequence (Solomon *et al.* 2004). Furthermore, another putative secreted protein, T19B10.2, was recently shown to be required for the osmotic resistance of *age-1* mutants (Lamitina and Strange 2005). Taken together, this evidence indicates that there may be a large number of yet unidentified proteins secreted from the hypodermis that are important for sensing external conditions and mediation of stress responses. Double-mutant analysis reveals that *osm-7, osm-11,* and *osr-1* may respond to different stimuli, but probably activate glycerol accumulation via a common downstream pathway.

If the defects in *osm-7* mutants are caused by constitutive signaling through osmotic stress response pathways, then it should be possible to restore osmotic sensitivity by elimination of downstream pathway members. The

unc-43/nsy-1/sek-1 MAPK pathway was previously shown not to be required for the acute osmotic stress resistance of *osr-1* animals (Solomon *et al.* 2004). In agreement with these results, we find that *unc-43*, *nsy-1*, *and sek-1* mutations do not suppress the Osr and high-glycerol phenotypes of *osm-7*. Thus, the *nsy-1* pathway is not required for the accumulation of glycerol that leads to resistance to acute osmotic stress. *nsy-1* pathway mutations were reported to suppress the chronic osmotic stress resistance of *osr-1* mutants (Solomon *et al.* 2004), but we find that the resistance of *osm-7* is not affected by the same mutations. This indicates that the *nsy-1* pathway is not required for the chronic Osr phenotype of *osm-7*, but given that the mechanism of chronic osmotic stress resistance is not understood, this result is difficult to interpret. It is possible that glycerol accumulation alone is sufficient for survival of chronic osmotic stress; indeed, all Osr strains tested in this work show resistance to both acute and chronic stress. Alternatively, there may be distinct mechanisms that are required for survival after the acute phase of an osmotic shock. Future work, such as suppressor screens, should help clarify the relationship between acute and chronic stress survival and reveal how many genetic pathways contribute to these phenotypes.

We also looked for evidence of interaction between *osm-7* and PLCβ/PKC signaling, which acts in parallel to the Hog1 MAPK pathway for osmotic resistance in yeast. Double mutants between *osm-7* and any of *egl-8* (PLCβ), *tpa-1*, *pkc-1*, or *pkc-2* had the same phenotype as the *osm-7* single mutant. This indicates that these genes are not required for signaling downstream of *osm-7*, but it remains possible that this pathway functions redundantly in parallel to the *nsy-1/sek-1* pathway or another unidentified pathway. In addition, *C. elegans* has four PKC homologs, whereas yeast only has one, and thus it is possible that they have redundant functions in worm. Based on the situation in yeast, it is likely that the pathways regulating the response to changing osmotic conditions are complex and overlapping. The Osr mutants provide a model for the dissection of these pathways in a multicellular organism and thus should provide insight into mechanisms of stress resistance with future study.

We thank K. Mase and M. Koga for sharing information prior to publication and members of the Thomas lab and Seattle worm community for helpful discussion. Aubrey Pullman provided assistance with graphics for figures. This work was supported by a grant from the National Institutes of Health.

LITERATURE CITED

Albertyn, J., S. Hohmann, J. M. Thevelein and B. A. Prior, 1994 GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. **14:** 4135–4144.

Alonso-Monge, R., E. Real, I. Wojda, J. P. Bebelman, W. H. Mager *et al.*, 2001 Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects. Mol. Microbiol. **41:** 717–730.

Brewster, J. L., T. de Valoir, N. D. Dwyer, E. Winter and M. C. Gustin, 1993 An osmosensing signal transduction pathway in yeast. Science **259:** 1760–1763.

Burg, M. B., E. D. Kwon and D. Kultz, 1997 Regulation of gene expression by hypertonicity. Annu. Rev. Physiol. **59:** 437–455.

Culotti, J. G., and R. L. Russell, 1978 Osmotic avoidance defective mutants of the nematode *Caenorhabditis elegans*. Genetics **90:** 243–256.

Dal Santo, P., M. A. Logan, A. D. Chisholm and E. M. Jorgensen, 1999 The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. Cell **98:** 757–767.

de Nobel, H., C. Ruiz, H. Martin, W. Morris, S. Brul *et al.*, 2000 Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. Microbiology **146:** 2121–2132.

Fraser, A. G., R. S. Kamath, P. Zipperlen, M. Martinez-Campos, M. Sohrmann *et al.*, 2000 Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature **408:** 325–330.

Garner, M. M., and M. B. Burg, 1994 Macromolecular crowding and confinement in cells exposed to hypertonicity. Am. J. Physiol. **266:** C877–C892.

Gustin, M. C., J. Albertyn, M. Alexander and K. Davenport, 1998 MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **62:** 1264–1300.

Hohmann, S., 2002 Osmotic stress signaling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. **66:** 300–372.

Iwasaki, K., D. W. Liu and J. H. Thomas, 1995 Genes that control a temperature-compensated ultradian clock in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **92:** 10317–10321.

Kim, D. H., R. Feinbaum, G. Alloing, F. E. Emerson, D. A. Garsin *et al.*, 2002 A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. Science **297:** 623–626.

Lamitina, S. T., and K. Strange, 2005 Transcriptional targets of DAF-16 insulin signaling pathway protect *C. elegans* from extreme hypertonic stress. Am. J. Physiol. Cell. Physiol. **288:** C467–C474.

Lamitina, S. T., R. Morrison, G. W. Moeckel and K. Strange, 2004 Adaptation of the nematode *Caenorhabditis elegans* to extreme osmotic stress. Am. J. Physiol. Cell. Physiol. **286:** C785–C791.

Lin, H., P. Nguyen and A. Vancura, 2002 Phospholipase C interacts with Sgd1p and is required for expression of GPD1 and osmoresistance in *Saccharomyces cerevisiae*. Mol. Genet. Genomics **267:** 313–320.

McMahon, L., J. M. Muriel, B. Roberts, M. Quinn and I. L. Johnstone, 2003 Two sets of interacting collagens form functionally distinct substructures within a *Caenorhabditis elegans* extracellular matrix. Mol. Biol. Cell **14:** 1366–1378.

Miller, K. G., M. D. Emerson and J. B. Rand, 1999 Goα and diacylglycerol kinase negatively regulate the Gqα pathway in *C. elegans*. Neuron **24:** 323–333.

Novelli, J., A. P. Page and J. Hodgkin, 2006 The C terminus of collagen SQT-3 has complex and essential functions in nematode collagen assembly. Genetics **172:** 2253–2267.

Remize, F., L. Barnavon and S. Dequin, 2001 Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. Metab. Eng. **3:** 301–312.

Robatzek, M., and J. H. Thomas, 2000 Calcium/calmodulin-dependent protein kinase II regulates *Caenorhabditis elegans* locomotion in concert with a G_o/G_q signaling network. Genetics **156:** 1069–1082.

Sagasti, A., N. Hisamoto, J. Hyodo, M. Tanaka-Hino, K. Matsumoto *et al.*, 2001 The CaMKII UNC-43 activates the MAPKKK NSY-1 to execute a lateral signaling decision required for asymmetric olfactory neuron fates. Cell **105:** 221–232.

Siderius, M., O. Van Wuytswinkel, K. A. Reijenga, M. Kelders and W. H. Mager, 2000 The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature. Mol. Microbiol. **36:** 1381–1390.

Solomon, A., S. Bandhakavi, S. Jabbar, R. Shah, G. J. Beitel *et al.*, 2004 *Caenorhabditis elegans* OSR-1 regulates behavioral and physiological responses to hyperosmotic environments. Genetics **167:** 161–170.

Stein, L. D., Z. Bao, D. Blasiar, T. Blumenthal, M. R. Brent *et al.*, 2003 The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. PLoS Biol. **1:** E45.

Tabuse, Y., 2002 Protein kinase C isotypes in *C. elegans*. J. Biochem. **132:** 519–522.

Take-Uchi, M., M. Kawakami, T. Ishihara, T. Amano, K. Kondo *et al.*, 1998 An ion channel of the degenerin/epithelial sodium channel superfamily controls the defecation rhythm in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **95:** 11775–11780.

Watts, J. L., E. Phillips, K. R. Griffing and J. Browse, 2003 Deficiencies in C20 polyunsaturated fatty acids cause behavioral and developmental defects in *Caenorhabditis elegans fat-3* mutants. Genetics **163:** 581–589.

Winter, A. D., and A. P. Page, 2000 Prolyl 4-hydroxylase is an essential procollagen-modifying enzyme required for exoskeleton formation and the maintainance of body shape in the nematode *Caenorhabditis elegans*. Mol. Cell. Biol. **20:** 4084–4093.

Communicating editor: B. J. Meyer