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The ERK MAP Kinase Cascade Mediates Tail Swelling and a Protective Response to Rectal Infection in *C. elegans*

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Summary

The nematode *Caenorhabditis elegans* is proving to be an attractive model organism for investigating innate immune responses to infection [1]. Among the known pathogens of *C. elegans* is the bacterium *Microbacterium nematophilum*, which adheres to the nematode rectum and postanal cuticle, inducing swelling of the underlying hypodermal tissue and causing mild constipation [2]. We find that on infection by *M. nematophilum*, an extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase cascade mediates tail swelling and protects *C. elegans* from severe constipation, which would otherwise arrest development and cause sterility. Involvement in pathogen defense represents a new role for ERK MAP kinase signaling in this organism.

Results and Discussion

The role of the ERK MAP kinase cascade in *C. elegans* development is well established [3]. In particular, ERK signaling has been shown to be necessary for the division and differentiation of the three hypodermal cells that form the vulva and has been studied in great detail in this context. Reduction of signaling gives rise to a vulvaless (Vul) phenotype as the hypodermal cells fail to differentiate. Conversely, constitutive activation of the ERK cascade causes additional hypodermal cells to take on vulval fates, giving rise to ectopic vulvae, the multivulva (Muv) phenotype. In certain cases, hyperactivation of the ERK pathway also causes a previously unexplained hermaphrodite tail abnormality [4, 5] (Figures 1A and 1B). The striking similarity between this tail abnormality and the deformation of the anal region (Dar phenotype) induced by infection with *M. nematophilum* (Figure 1C) suggested that the ERK MAP kinase cascade might participate in the response of *C. elegans* to infection.

We found that this cascade is indeed required for tail swelling. The core components of the cascade are the MAP kinase kinase kinase LIN-45 Raf, the MAP kinase kinase MEK-2, and the MAP kinase MPK-1. Reduction of function mutants for each of these kinases (*lin-45(sy96)* [6], *mek-2(n1989)* [7], and *mpk-1(ku1)* [8]) were tested for response to the pathogen. Unlike wild-type animals, which display the Dar phenotype within hours of exposure to *M. nematophilum*, these mutants failed to swell, exhibiting instead a bacterially unswollen (Bus) phenotype (Figure 1D). In order to exclude the possibility that this resulted simply from an absence of infection, the animals were stained with the nucleic acid dye SYTO 13, which allows visualization of bacteria adherent to the rectum. The presence of rectal staining in each of these strains confirms infection.

In addition to genetic ablation of the ERK MAP kinase cascade, we tested the effect of chemically inhibiting the pathway using the compound U0126, which specifically blocks the activity of MEK-2 [9]. Wild-type worms grown in liquid culture containing 50 μM U0126 failed to swell in the presence of the pathogen despite the establishment of infection (Figures 1E and 1F). Signaling through the ERK MAP kinase pathway is necessary, therefore, for the response of *C. elegans* to infection by *M. nematophilum*.

Many of the factors known to function upstream and downstream of the core kinases appear, however, not to be involved in this response. In the worm, the ERK MAP kinase cascade is normally activated by the Ras protein LET-60 in response to signaling from the epidermal and fibroblast growth factors LIN-3, EGL-17, and LET-756. Other upstream and accessory factors include the receptors LET-23 and EGL-15, the SH domain-containing protein SEM-5, the guanine nucleotide exchange factor SOS-1, the scaffold proteins KSR-1 and KSR-2, the protein phosphatase 2A subunit SUR-6, the Ras binding protein SOC-2, and the cation diffusion facilitator CDF-1. Downstream of the cascade are the transcriptional regulators LIN-1, LIN-31, EOR-1, EOR-2, SUR-2, and LIN-25. Strains carrying reduction of function mutations in all of these genes were tested for response to *M. nematophilum*. Although most of these mutants responded as wild-type with postanal swelling, *ksr-1(n2682)* [10], *sur-2(ku9)* [11], and *lin-25(e1446)* [12] mutants failed to swell.

Surprisingly, strains carrying both loss- and gain-of-function mutations in LET-60 Ras behave like wild-type with respect to tail-swelling. The ERK MAP kinase cascade may, therefore, be activated in a Ras-independent manner in the tail following infection with *M. nematophilum*, in contrast to the Ras dependence of its activation in other settings. However, as the mutant alleles tested were necessarily not null (since complete loss of function of the ERK MAP kinase cascade is lethal), the involvement of LET-60 and other upstream proteins cannot yet be rigorously excluded. Nevertheless, the observations suggest that only a subset of the factors associated with ERK signaling in other contexts are relevant in the swelling response to infection (Figure 1I).

The established role of the ERK MAP kinase cascade in the specification of cell fates during worm development raises the possibility that the failure of the mutants described above to swell in response to infection reflects a developmental defect. We have found that expression of two hindgut cell markers, *egl-5* and *mab-9*, is unaltered in the rectal cells of the *mpk-1(ku1)* mutant (data not shown), suggesting that the development of

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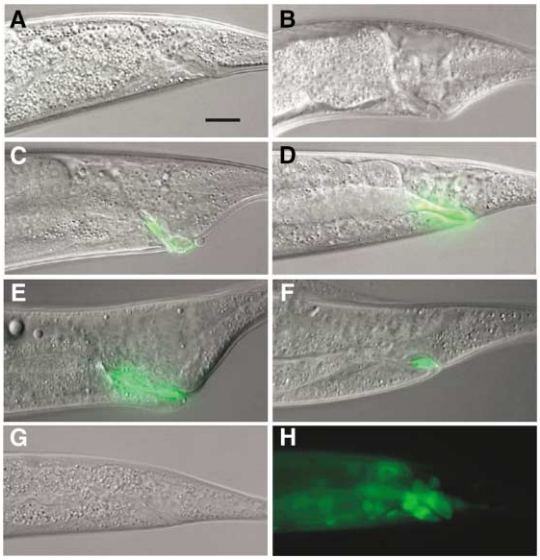

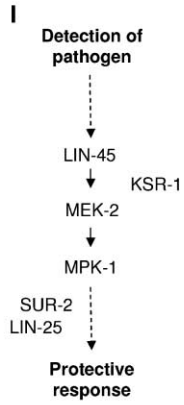

Figure 1. The ERK MAP Kinase Cascade Mediates *C. elegans* Tail Swelling in Response to Infection by *M. nematophilum*

(A–F) The nucleic acid stain SYTO 13 highlights the infecting bacteria (green fluorescence) in Nomarski images of adult *C. elegans* tails.

(A) Uninfected wild-type.

(B) Tail abnormality of uninfected worm carrying insertion *gals37* [hs-*mpk-1*, EF1α-Dsor1, *unc-30*] [31] after heat shock. Dsor1 is the *Drosophila* ortholog of the ERK MAP kinase kinase MEK-2. This phenotype is also seen after heat shock of strains carrying *eEx544* [pPD49.83su1 [32], *rol-6(dm)*] (from which a gain-of-function form of Dsor1 is expressed), and *kuIs23* [hs-*mek-2*S223ES227D [4], *rol-6(dm)*] (encoding a MEK-2 gain-of-function protein). Strains carrying *eEx548* [*lin-45*S312AS453A [5], *rol-6(dm)*] (encoding a gain-of-function form of LIN-45) and heat-shocked strains bearing *eEx539* [pMS88 *raf(gf)* [33], *rol-6(dm)*] (in which the kinase domain of the *Drosophila* ortholog of LIN-45 is fused to the transmembrane domain of the Torso receptor) also show a similar defect.

(C) Dar phenotype of infected wild-type.

(D) Bus phenotype of infected *mpk-1(ku1)*.

(E and F) Wild-type worms infected after growth in liquid culture in 10% *M. nematophilum*, 90% HB101 in the presence of 0.5% v/v DMSO display the Dar phenotype in the absence of the MEK-2 inhibitor U0126 (E) or the Bus phenotype in the presence of U0126 (F).

(G and H) L4 stage DA1783 hermaphrodite bearing an integrated *mpk-1::GFP* fusion construct. The rectum is visible in the Nomarski image (G) while the fluorescence image of the same animal (H) shows expression of MPK-1::GFP in the cells lining this structure. Scale bar in (A) represents ~35 μm for (A)–(H).

(I) Schematic representation of the subset of ERK MAP kinase signaling components identified as mediators of tail swelling. Both the mechanism of pathogen detection and the output of the cascade are, as yet, unknown.

the hermaphrodite hindgut is not compromised by this decrease in MAP kinase signaling. Misspecification of one hindgut cell, hyp12, has been reported in certain MAP kinase signaling mutants (for example, [13]). This defect does not, however, correlate with the observed failure to swell in response to infection. For instance, it is observed in only 6% of *sur-2(ku9)* animals [14], while the Bus phenotype is 100% penetrant in this strain. We propose, therefore, that ERK signaling plays an instructive, rather than permissive, role in tail swelling. The observed induction of the Dar phenotype through the activation of the MAP kinase pathway in the absence of infection, as described above, is supportive of this notion. Expression of the pathway components MPK-1, KSR-1, and SUR-2 in the worm hindgut throughout larval development and in adulthood confirms the potential for the MAP kinase pathway to be activated in direct response to infection (Figures 1G and 1H). Such a role in nematode response to environmental stimuli is not unprecedented; ERK signaling mediates perception and transmission of sensory signals in olfactory neurons [15]. Activation of the *C. elegans* MAP kinase pathway also promotes protein degradation in muscle cells [16], another nondevelopmental function.

In addition to tail swelling, infection with *M. nematophilum* also causes constipation [2]. The posterior portion of the intestinal lumen becomes noticeably distended when wild-type worms are infected (Figures 2A and 2B). This effect is markedly exacerbated in the absence of a functional ERK MAP kinase cascade. Wild-type worms and strains carrying mutations in each of the genes found to be necessary for the swelling response, *lin-45*, *mek-2*, *mpk-1*, *ksr-1*, *sur-2*, and *lin-25*, do not display constipation when grown on the standard *Escherichia coli* strain OP50. In contrast, when grown

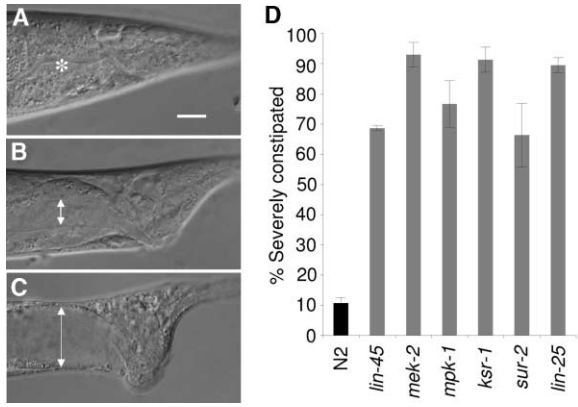

Figure 2. The ERK MAP Kinase Cascade Protects against Severe Constipation

(A) Uninfected wild-type adult. An asterisk indicates the intestine.

(B and C) Infection by *M. nematophilum* causes mild (B) or severe (C) constipation in wild-type worms. The extent of distension of the intestinal lumen is indicated by double-headed arrows. Scale bar in (A) equals ~35 μm for (A)–(C).

(D) Mean percentage of animals suffering severe constipation in wild-type (black bar) and ERK MAP kinase mutant (gray bars) populations grown on OP50 mixed with *M. nematophilum*. Standard errors are indicated. Constipation was not observed in uninfected control populations grown on OP50 alone. Strains: N2 wild-type, *lin-45(sy96)*, *mek-2(n1989)*, *mpk-1(ku1)*, *ksr-1(n2682)*, *sur-2(ku9)*, and *lin-25(e1446)*.

on lawns containing *M. nematophilum*, animals can become severely constipated, often with apparently complete blockage of the rectum (Figure 2C). In wild-type populations, only 11% suffer this fate, while in the MAP kinase cascade mutant populations, the majority of worms do, indicating that these mutants are hypersensitive to infection. The penetrance of this effect varies between strains from 66% (*sur-2(ku9)*) to 93% (*mek-2(n1989)*) (Figure 2D). Since, as noted above, some of the tested mutant alleles are not null, the differences in susceptibility can be attributed to the relative strengths of the mutations or may also be indicative of partial redundancies.

From the increase in susceptibility to constipation observed in ERK MAP kinase cascade mutants, we conclude that in wild-type worms this cascade limits the severity of constipation caused by *M. nematophilum* infection. This limitation serves the important function of preventing developmental arrest and sterility. Strongly constipated wild-type worms bear, on average, only 10 ± 2 (n = 18) progeny, much less than the average of 107 ± 9 (n = 11) progeny borne by their mildly constipated counterparts, which, in turn, is many fewer than the 216 ± 8 (n = 9) progeny produced by uninfected wild-type hermaprodites. The deleterious effect of constipation is yet more conspicuous in the ERK MAP kinase mutant *mpk-1(ku1)*. Uninfected and mildly constipated *mpk-1(ku1)* animals have broods of 105 ± 12 (n = 10) and 61 ± 8 (n = 10), respectively. The majority (79%) of infected *mpk-1(ku1)* animals suffer severe constipation, and of these, 45% (n = 141) fail to reach adulthood and hence bear no young. The remainder that do eventually progress through the final larval moult are largely sterile, with average broods numbering only 3 ± 1 (n = 11). Considering the relative proportions of wild-type and *mpk-1(ku1)* mutant animals that suffer severe constipation on infection, infected wild-type populations are three times more successful reproductively than infected *mpk-1(ku1)* mutant populations, when compared with uninfected populations of the same strains. These serious consequences of infection in the absence of ERK signaling indicate that activation of this MAP kinase pathway is a protective response to infection. The postanal swelling described earlier, which is also mediated by this cascade, may constitute part of the protective response. Swelling distorts the rectal anatomy and may thereby limit the extent of infection or relieve constipation.

In higher eukaryotes including *C. elegans*, cascades analogous to the ERK MAP kinase pathway activate two additional types of MAP kinase: p38 and c-Jun N-terminal kinase (JNK). A kinase of the former class, PMK-1, has recently been shown to mediate an innate immune function in *C. elegans*, providing defense against intestinal infection by *Pseudomonas aeruginosa* [17], *Staphylococcus aureus* [18], and *Salmonella enterica* [19]. Strains carrying mutations in the genes encoding the MAP kinase kinase kinase NSY-1 and the MAP kinase kinase SEK-1, which function upstream of PMK-1, were tested for response to *M. nematophilum*. Like wild-type, these mutants developed postanal swelling. Although the JNK pathway has not been implicated in defense in *C. elegans*, it has established roles in immunity in other

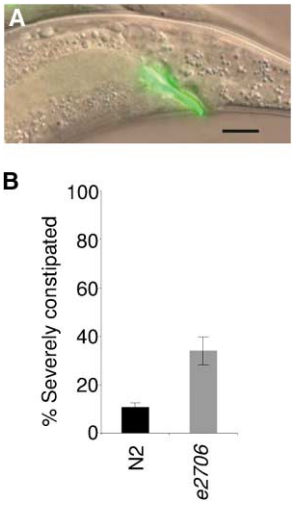

Figure 3. *bus(e2706)* Mutants Fail to Swell on Exposure to *M. nematophilum* and Are Hypersensitive to Infection

(A) Bus phenotype of infected *bus(e2706)* adult. Scale bar represents ~35 μm.

(B) Mean percentage of animals suffering severe constipation in wild-type (black bar) and *bus(e2706)* mutant (gray bar) populations grown on OP50 mixed with *M. nematophilum*. Standard errors are indicated. Constipation was not observed in uninfected control populations grown on OP50 alone.

organisms [20]. Null mutations of the JNK kinase, JNK-1, or one of the upstream MAP kinase kinases, MEK-1, do not alter the response of *C. elegans* to infection with *M. nematophilum*. The ERK MPK-1, therefore, appears to be unique among the *C. elegans* MAP kinases in mediating the response to rectal infection by this pathogen. These results show that the worm uses distinct signaling pathways to mount appropriate responses to different kinds of infection, as also indicated by other recent observations [21].

In order to identify factors necessary for infection by *M. nematophilum* and response thereto, screens have been performed for *C. elegans* mutants altered in response to infection. These screens have identified 20 *bus* loci to date which, when mutated, prevent either the establishment of infection or the subsequent swelling response (M. Gravato-Nobre, H.R.N., R. Nijland, D. O'Rourke, D. Whittington, K.J. Yook, and J.H., unpublished data). One mutant to which bacteria adhere but fail to induce swelling is *bus(e2706)* (Figure 3A). When exposed to the pathogen, as compared with wild-type, three times as many *bus(e2706)* animals become severely constipated (Figure 3B). The resemblance of these mutant phenotypes to those of the ERK MAP kinase mutants suggests the gene may be involved in the MAP kinase cascade. To assess this possibility, the gain-of-function form of MEK-2, MEK-2 S223ES227D [4], was overexpressed in the *bus(e2706)* mutant. While such overexpression causes massive swelling of the tail region in wild-type animals, in the mutant background the severity of this effect is reduced (Figures 4A and 4B). In contrast to this partial suppression by *bus(e2706)*, MEK-2 S223ES227D-mediated tail swelling is completely suppressed by *mpk-1(ku1)* (Figure 4C),

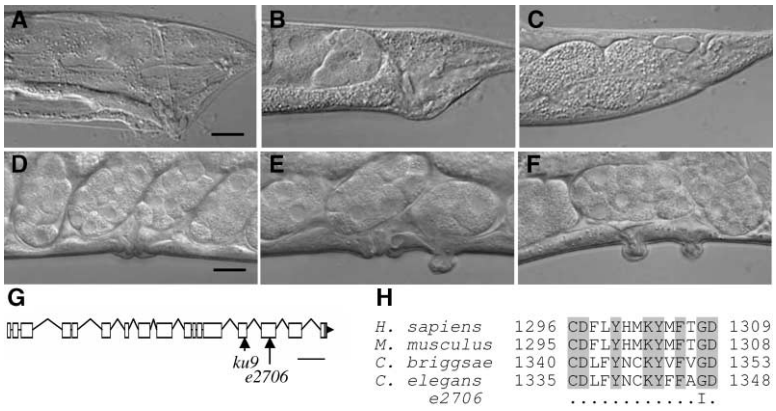

Figure 4. *e2706*, which Partially Suppresses ERK MAP Kinase-Mediated Tail Swelling and Causes Defects in Vulval Development, Is a Missense Allele of *sur-2*

(A–C) Overexpression of MEK-2 S223E227D causes a Dar phenotype in wild-type (A), which is partially suppressed by *bus(e2706)* (B) and completely suppressed by *mpk-1(ku1)* (C).

(D) Wild-type vulva.

(E and F) Hyperinduction of vulval hypodermal cells in *let-23(n1045)* (E) and *bus(e2706)* (F). Scale bar in (A) equals ~35 μm for (A)–(C) and in (D) equals ~25 μm for (D)–(F).

(G) Schematic representation of the *sur-2* gene. Scale bar equals ~1 kb. The *ku9* allele, as shown in Figure 2, is a C to T transition in exon 14, resulting in a change from arginine to an opal stop codon [11]. The new allele, *e2706*, identified on the basis of the Bus phenotype, is a GG to AT change in exon 15.

(H) The *e2706* allele causes the substitution of an isoleucine residue in the place of glycine at position 1347 of the SUR-2 protein. This residue falls within one of the conserved homology blocks of this protein as is illustrated through alignments with the human, mouse, and *C. briggsae* orthologs.

sur-2(ku9), or *lin-25(ar90)*, suggesting that *e2706* is weaker than these other ERK pathway mutant alleles. Consistently, the proportion of *bus(e2706)* populations suffering from severe constipation (34%) is not as great as that of the other ERK MAP kinase cascade mutants (66%–93%).

As mentioned, the ERK MAP kinase cascade is essential for vulval development. Reduction of ERK MAP kinase signaling can cause hyperinduction of vulval hypodermal cells [22] (Figures 4D and 4E). At 15°C, 11% (n = 122) of *bus(e2706)* worms display such a mutant phenotype, suggesting that the gene might play a role in the vulval aspect of ERK function (Figure 4F). Indeed, *bus(e2706)* suppresses the penetrance of the Muv phenotype of the *let-60* gain-of-function allele *n1046* [23] from 92% (n = 282) to 26% (n = 370) at 20°C, confirming such a role.

snipSNP mapping [24] placed *bus(e2706)* on the right arm of chromosome I. *sur-2*, encoding a component of the Mediator complex that functions in transcription downstream of ERK signaling, also maps to this region, suggesting that *e2706* might be a weak allele of *sur-2*. In support of this, *e2706* fails to complement *sur-2(ku9)* for the Bus phenotype. Furthermore, the *e2706* Bus phenotype is rescued by extrachromosomal arrays containing an 11.6 kb PCR product comprising the *sur-2* open reading frame and ~0.5 kb upstream and downstream thereof. Sequencing of *e2706* revealed the tandem mutation of adjacent guanine residues in exon 15 of the *sur-2* gene, causing the substitution of an isoleucine residue in place of glycine at position 1347 of the encoded protein (Figures 4G and 4H). As shown in Figure 4, this residue is conserved in a number of species. It falls within one of the two domains defined in the proposed *Saccharomyces cerevisiae* ortholog, Gal11, as being essential for activity [25]. The *e2706* mutation is likely, therefore, to reduce the transcriptional output of ERK signaling.

This work has defined a new function for the well-studied ERK MAP kinase pathway in *C. elegans* and has identified a novel allele of one of its components, *sur-2*. We have shown that the ERK MAP kinase cascade, already known to act in both plant [26] and mammalian [27, 28] defense, protects *C. elegans* against the consequences of a bacterial infection. This demonstration is consistent with broad evolutionary conservation of a role for ERK MAP kinase cascades in innate immunity.

Experimental Procedures

Infection and Staining

Bacterial strains were grown in LB medium at 37°C to an OD_{600} of 0.25 (~24 hr) or 0.15 (~48 hr) for OP50 and *M. nematophilum*, respectively. NGM plates were seeded with either OP50 alone or a trace (usually 0.01% (v/v)) of *M. nematophilum* in OP50. *C. elegans* strains were cultured on these plates at 25°C. Infected animals were washed off in 10 ml Tris-buffered saline (TBS) and allowed to settle for 60 min to complete digestion of bacteria in the gut. 60 μl of settled worms were added to 300 μl diluted SYTO 13 live-cell nucleic acid stain (Molecular Probes, 15 μM in TBS) and left in the dark for 60 min. 3 μl of settled, stained worms were then added to 3 μl 4% propylene phenoxytol in M9 and viewed using Nomarski microscopy with UV fluorescence.

Liquid Culture and Inhibition Using U0126

Gravid wild-type hermaphrodite worms were treated with alkaline hypochlorite, and the released embryos were grown to the first larval stage (L1) by shaking for 18 hr in M9 at 25°C. The starved L1s were transferred to S medium containing *E. coli* strain HB101 at a concentration of 3 g/100 ml and grown to the L3 stage at 25°C. The culture was then divided among six flasks, and 0.3 g/100 ml *M. nematophilum* was added to three of them. The MEK-2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene (Sigma U0126, 10 mM in DMSO) was added at a final concentration of 50 μM to one of each type of culture. As negative controls, DMSO (0.5% v/v) alone was added to another of each type of culture and no additions were made to a third pair of cultures. The cultures were grown for a further 48 hr at 25°C, with 200 rpm shaking, after which time a 5 ml sample was taken from each culture and added to 5 ml TBS then stained using the SYTO 13 live-cell nucleic acid stain as described above. Additional experiments exposed animals to *M. nematophilum* and the inhibitor directly from the L1 stage.

Reporter Constructs

The expression patterns of two *egl-5* reporters were examined in the *mpk-1(ku1)* mutant background. The first, EM#278, contains 13.2 kb upstream of the *egl-5* open reading frame and *egl-5* coding sequence up to the middle of exon 3, joined in frame to the *GFP* cassette from plasmid pPD96.67 [29]. The second, pLG7, contains a 1.4 kb fragment of the *egl-5* promoter, which directs expression

in, among others, the rectal cells B, F, U, K, and P12.p (L. Girard, personal communication), inserted upstream of the *pes-10* minimal promoter in vector pPD107.94 that contains an NLS-GFP-lacZ reporter. Expression of *mab-9* was examined using the pAW118 rescuing *mab-9::GFP* fusion construct [30]. Expression of *mpk-1* was observed in strain DA1783, which contains an integrated transgene consisting of a PCR product spanning ~4 kb upstream of the *mpk*-1 open reading frame, with *GFP* fused to the 3′ end (Y. You, personal communication). Expression of *ksr*-1 was observed in strain UP442, which bears an integrated array containing the rescuing *ksr-1::GFP* fusion construct pMS157 (M. Sundaram, personal communication). To examine *sur-2* expression, genomic DNA from ~0.5 kb upstream of the *sur-2* open reading frame to the middle of the fourth exon was amplified by PCR and subcloned in frame with *GFP* in vector pPD96.04. The resulting plasmid, pHN2, was injected into N2 worms along with the marker pCes1943, and stable Rol lines were obtained.

Scoring Constipation

Gravid hermaphrodites were treated with alkaline hypochlorite and the released eggs were transferred to plates seeded with either OP50 or OP50 and *M. nematophilum* as described above. Plates were scored after 3 or 4 days incubation at 25°C for OP50 and mixed bacterial lawns, respectively. The fraction of some mutant populations that arrests during larval development with a characteristic rod-like morphology was excluded from all scores. Worms were graded as not constipated, mildly constipated, or severely constipated, according to observation by dissecting microscope. Samples from each category were mounted for viewing by Nomarski microscopy to verify scoring. Multiple trials were done for each strain (to a total of at least 200 animals), and the mean percentage showing severe constipation was calculated. A representative group of strains were scored blind to ensure objectivity.

Brood Counts

Eggs released from the alkaline hypochlorite treatment of gravid wild-type and *mpk-1(ku1)* mutant hermaphrodites were transferred to plates seeded with OP50 or OP50 and *M. nematophilum*, as described above, and incubated at 25°C. Single hermaphrodites were picked the following day to similarly seeded plates and transferred to fresh plates on subsequent days until no more eggs were laid. Hatched progeny on all plates were then counted and summed. Mean brood sizes and standard errors were calculated.

For those grown on OP50 and *M. nematophilum*, the singled hermaphrodites were scored on the fourth day after bleaching as mildly or severely constipated. Many infected animals were singled in order to ensure the availability of at least 10 broods from each category.

To assess development, severely constipated animals were viewed using Nomarski microscopy and scored for the presence of alae, an adult cuticular structure.

Heat Shock

The insertion *kuIs23* [4] contains a gene encoding the gain-of-function form of MEK-2, MEK-2 S223ES227D, under the control of the heat shock promoter *hsp16-41*, along with the transformation marker *rol-6*(dominant). This array was crossed in to the *mpk-1*, *sur-2*, *lin-25*, and *bus(e2706)* mutant backgrounds using the roller phenotype of *rol-6* to confirm the presence of the transgene. Transgenic animals were cultured at 20°C until the L3 stage, then heat shocked for 45 min at 37°C before being returned to 20°C to develop to adulthood. Adult animals were anesthetized in 4% propylene phenoxytol in M9 and mounted on agarose pads for Nomarski microscopy.

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