An Observational Analysis of *Saccharomyces cerevisiae* Infections in *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* is a model organism for investigating innate immunity using microbial pathogens. *Saccharomyces cerevisiae,* commonly known as baker's yeast, was found to associate with *C. elegans* by colonizing the intestinal lumen and causing intestinal distension. We found that when grown on *S. cerevisiae*, mutants carrying mutations in mitogen-activated protein (MAP) kinase kinase genes *mek-1* and *mek-2* accumulated intestinal *S. cerevisiae* more rapidly than wild type. Intact *S. cerevisiae* cells expressing an RFP-tagged protein were visible in the intestinal lumen by fluorescent microscopy, and their accumulation correlated with intestinal distension. The infection also disrupted egg laying behavior and promoted internal hatching of offspring, leading to matricidal death. Infected worms displayed increased lipofuscin in the intestinal cells compared to controls grown on *E. coli* OP50 or Heat Killed *E. coli* OP50. *S. cerevisiae* infected worms recovered from infection when transferred onto a freshly spotted *E. coli* OP50 plate.

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Introduction

Caenorhabitidis elegans are soil dwelling nematodes which feed on microbes and decaying matter (Worm Atlas, 2006). The average life span of a hermaphrodite worm is 21 days and adult hermaphrodites reach over 1mm in length (McGhee, 2007). Fig. 1 is a differential interference contrast (DIC) image of an adult hermaphrodite worm with its major body parts labeled (Fig. 1). The gut of *C. elegans* consists of 3 major structures: the pharynx, intestine, and the rectum (Wormatlas, 2004). The pharynx contains the pharyngeal grinder located in the terminal bulb and is responsible for crushing internalized microbes (Wormatlas, 2004). The mechanically disrupted food is then passed onto the intestinal tract for further digestion.

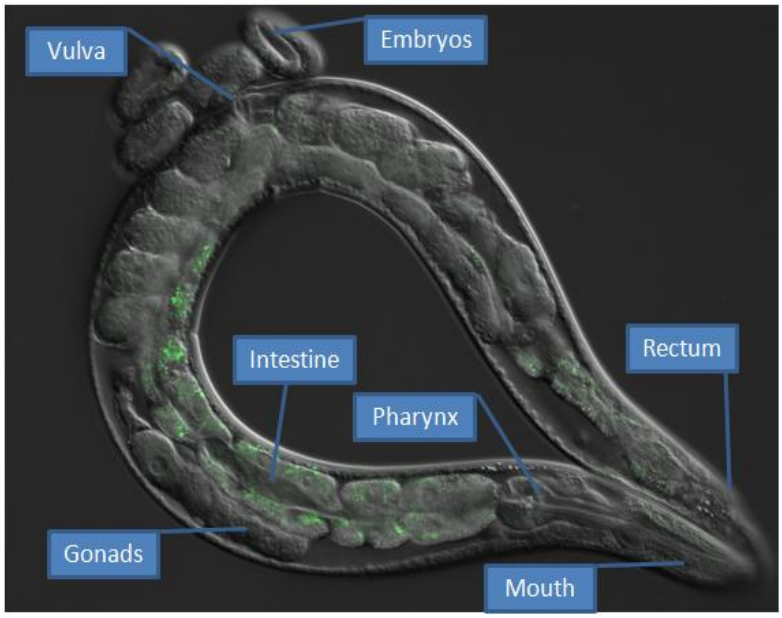

Fig.1 DIC image of an adult hermaphrodite *Caenorhabditis elegans* with labeled anatomy

Intestine of C. elegans

The intestine is composed of 20 individual cells which form pairs to construct intestinal ring structures called “int” rings (Wormatlas, 2004) (Fig. 2).

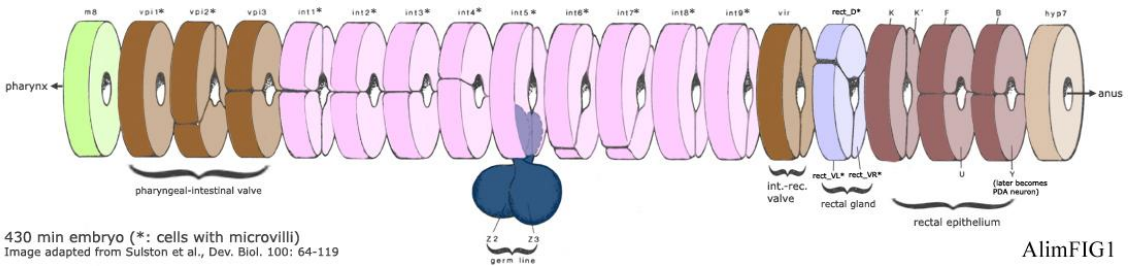

Fig.2 Structure of the Intestine (Worm Atlas, 2007)

The inside lining of the intestinal tract is covered with microvilli extending into the luminal space and is coated with a matrix of glycoproteins called the glycocalyx (Wormatlas, 2004) (Fig. 3).

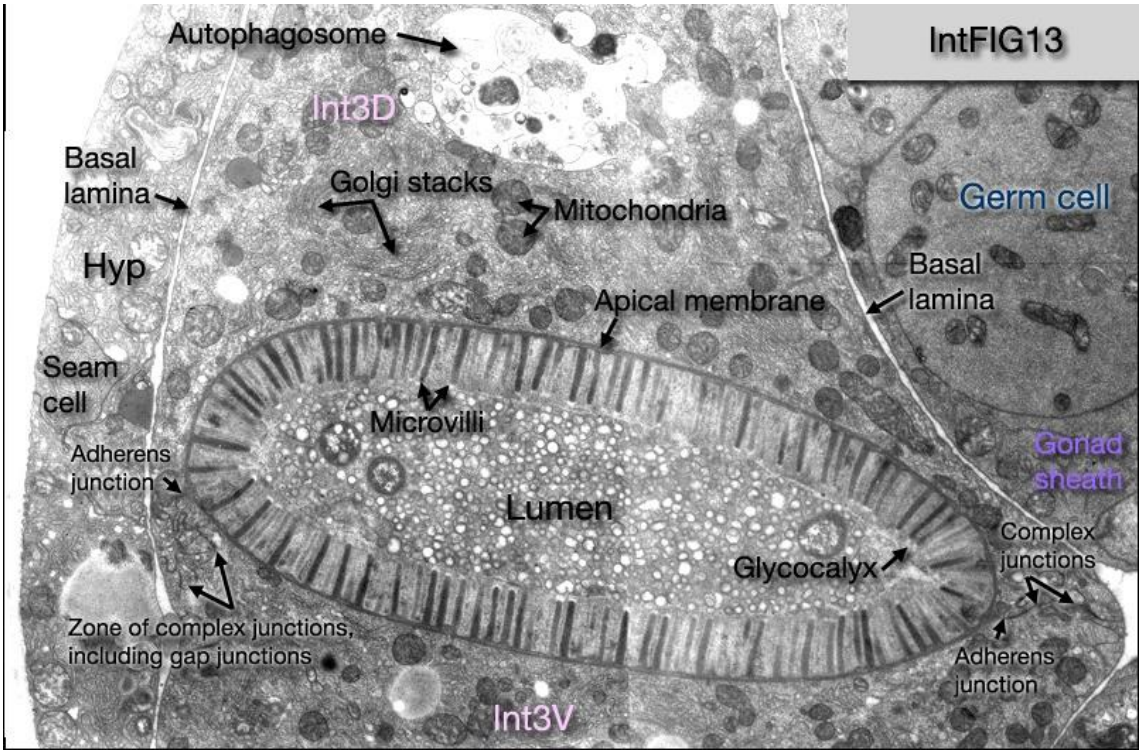

Fig.3 Transmission electron microscopy image of a cross section of a L4 hermaphrodite intestine (Worm Atlas, 2004) Hyp = Hypodermis cells, Int3D = Intestinal cell number 3, dorsal view, Int3V = Intestinal cell number 3. ventral view.

Life Cycle of C. elegans

The majority of *C. elegans* are hermaphrodites, but 0.01% of the population

are male (Worm Atlas, 2006). Hermaphrodites reproduce by self-fertilization and each hermaphrodite produces 200-300 offspring from the oocytes formed in the two arms of the female gonad (Wormatlas, 2006). An adult worm usually holds 8-12 embryos within the uterus at any given time (Worm Atlas, 2006). The embryos are laid periodically every 15-20 minutes when food is plentiful and worms are not starved (Worm Atlas, 2006). Hermaphrodites do not lay eggs in the absence of food, which can lead to matricidal death if eggs hatch internally (Worm Atlas, 2006).

Worms have a generation time of 3.5 days at 20°C (Worm Atlas, 2006). The postembryonic life cycle consists of passing through four developmental larval stages and a reproductive adult (Worm Atlas, 2006) (Fig. 4). In addition to these five stages, an alternative stress-resistant dauer larva substitutes for the L3 larvae in response to stressful environmental conditions experienced by the L1 and L2 larval stages (Hu, 2007; Wormatlas, 2006).

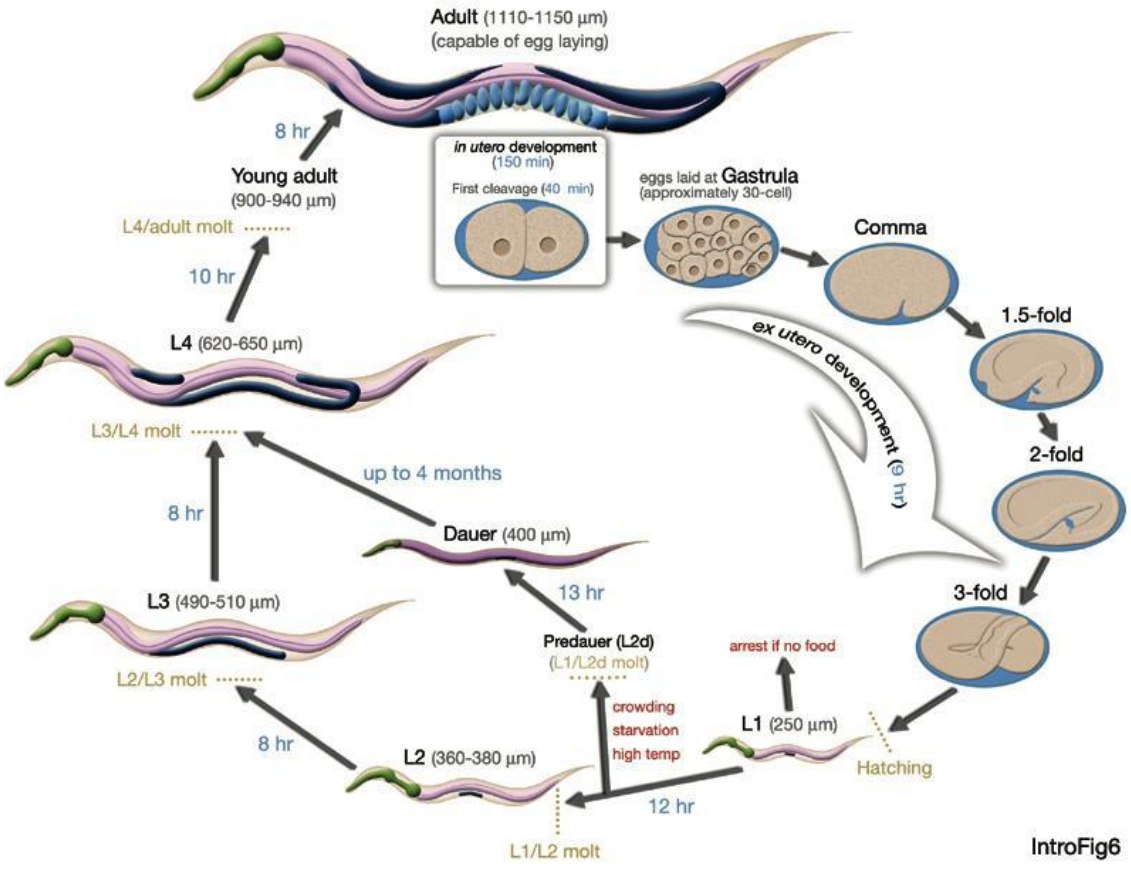

Fig.4 Developmental stages of *C. elegans* (McGhee, 2007)

Defecation Cycle of C. elegans

The worm eliminates ingested and digested materials through the rectum by

defecation (Worm Atlas, 2006). Defecation occurs in a stepwise fashion which constitutes contraction of specific regions of the intestine to produce a thrust of intestinal content out through the rectum (Liu and Thomas, 1994; C. elegans II, 1997). Defecation occurs periodically in ~ 45 seconds cycles (Liu and Thomas, 1994; C. elegans II, 1997).

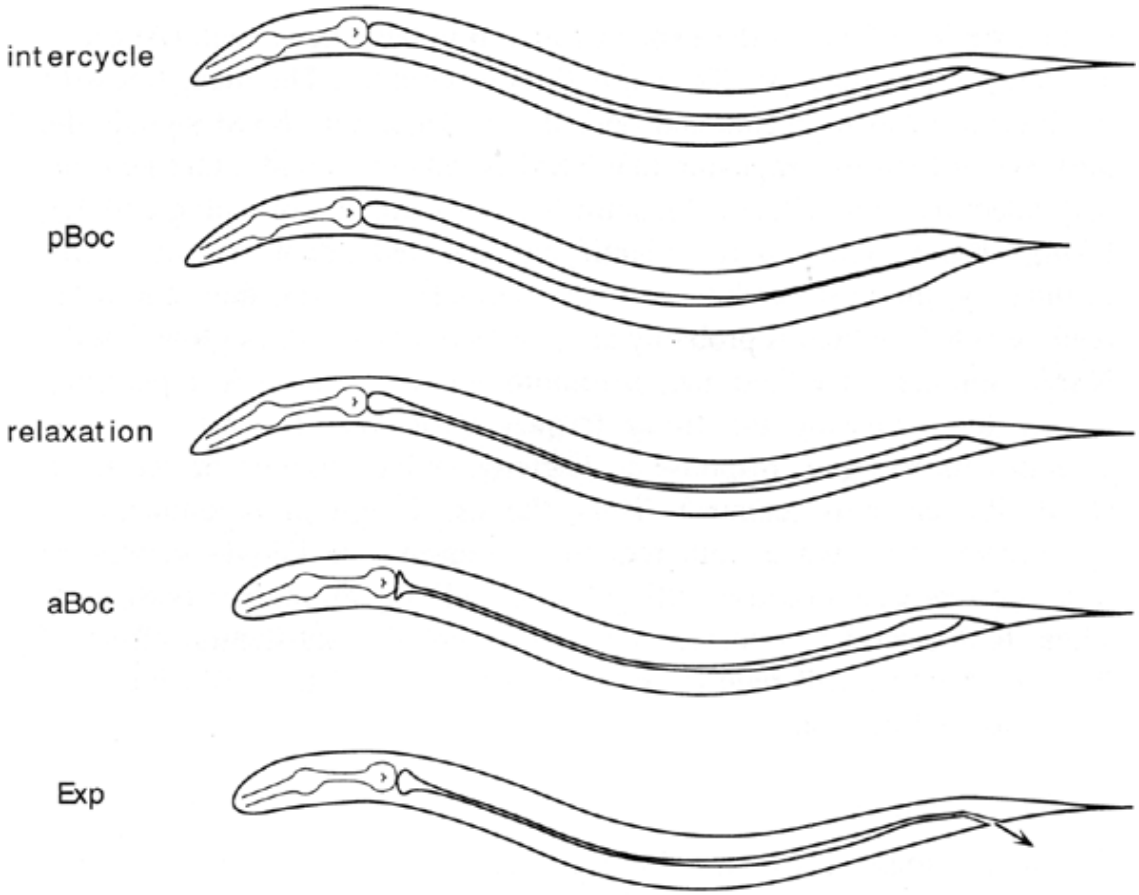

Fig.5 Movements during a defecation cycle (Thomas and Avery, 1997) The 5 movements of the defecation cycle. The **intercycle** is the resting phase. **pBoc** is the peak contraction of the posterior body muscle. In the pBoc the intestinal content is collected to the anterior end of the intestine. After pBoc, **Relaxation** occurs in the body muscle to prepare for the aboc. **aBoc** is the anterior body muscle contraction which results in intestinal contents to be collected at the preanal region. **Exp** is the final contraction of the enteric muscles (expulsion muscle contraction, EMC) resulting in an expulsion of intestinal contentment (Thomas and Avery, 1997).

C. elegans and Pathogenesis Models

Virulence of and host responses to many microbial pathogens have been studied in *C. elegans* including *Yersinia pestis* and *Microbacterium nematophilum (*Darby *et al.,* 2002; Joshua *et al.*, 2003; Darby *et al.,* 2007; Hogdkin *et al*., 2000). *Y.*

pestis, the bacterium that causes bubonic plague in humans, is pathogenic to *C. elegans (*Darby *et al.,* 2002; Joshua *et al.*, 2003). It produces biofims which adhere to and cover the mouth of the worm, blocking food entry and leading to starvation *(*Darby *et al.,* 2002; Joshua *et al.*, 2003). Intestinal infections of *C. elegans* by many pathogenic bacteria have been studied in which the organisms become pathogenic only after ingestion. Once in the gut they can accumulate, causing distension, damage to the intestinal epithelium, and death. Examples of pathogens that can cause such intestinal infections are *P. aeruginosa* PA14, *Salmonella typhimurium*, *Serratia marcescens*, and *Enterococcus faecalis* (Gravato-Nobre and Hodgkin 2005). *M. nematophilum* is a soil bacterium which adheres to and colonizes the rectum of the worm (Hodgkin *et al*., 2000). Colonization of the rectum blocks defecation and promotes constipation (Nicholas and Hodgkin, 2004). *M. nematophilum* infection was shown to induce an immune response characterized by a deformed anal region (DAR) phenotype, and requiring an ERK MAP kinase signaling pathway (Nicholas and Hodgkin, 2004). Colonization of the rectum in the absence of this response leads to severe constipation and decreased life span (Nicholas and Hodgkin, 2004). In work described here, we showed that infection with *S. cerevisiae* also induces this DAR phenotype in wild type *C. elegans* (Fig. 6).

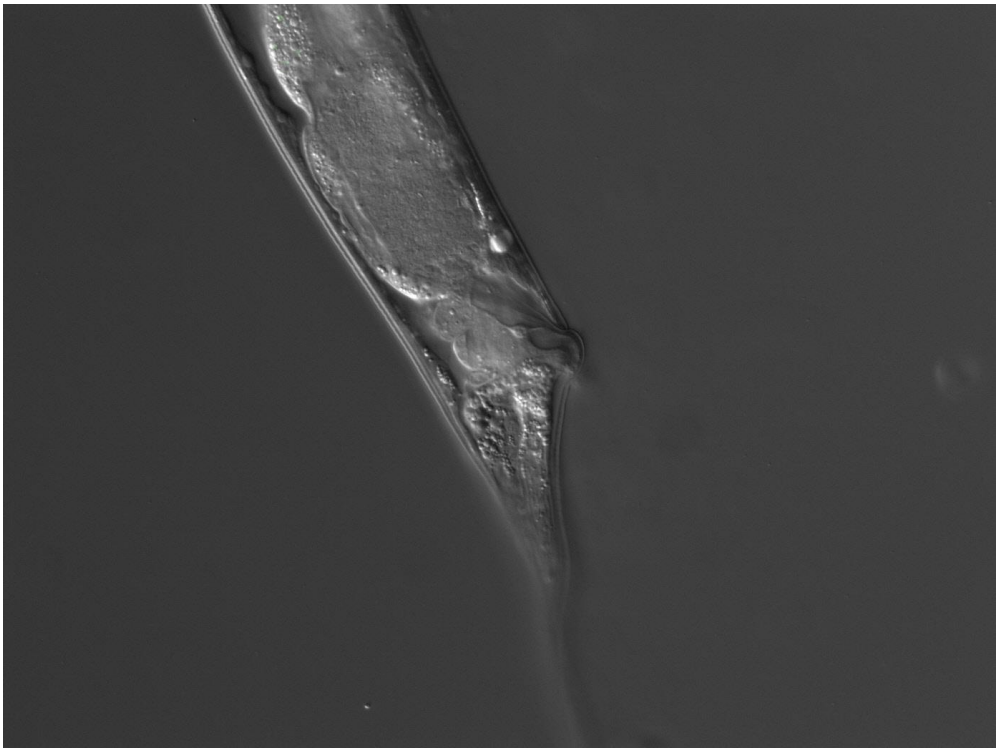

Fig.6 Deformed anal region (Dar) Phenotype in a *C. elegans* adult hermaphrodite resulting from interaction with a mixed lawn of *E. coli* OP50 and *S. cerevisiae.*

Lipofuscin

The intestine is responsible for secreting reactive oxygen species (ROS), digestive enzymes, and antimicrobial peptides to break down ingested microbes (Chávez *et al.,* 2007*)*. In parallel to secretion of ROS, the intestinal cells must protect themselves from these hazardous chemicals, and to do so, superoxide dismutase (SOD) is produced to neutralize the negative effects (Chávez *et al.,* 2007*)*.

Aging adult worms have an intestinal pigment similar to the dark brown pigmentation found consistently in other aging animals (Yin, 1996; Gerstbrein *et al.*, 2005). This brown pigmentation, called lipofuscin, results from accumulation of lipid-protein complexes formed from oxidized proteins and lipids through the action of free radicals and ROS (Terman *et al.,* 2006). Lipofuscin exhibits auto-fluorescence when excited with light ranging in the 400nm – 450nm spectrum (Gerstbrein *et al.*, 2005).

Signal Transduction Pathway

Several signaling pathways in *C. elegans* have been implicated as being important in resistance towards microbial pathogens and environmental stress (Koga *et al.*, 2000; Kim *et al.,* 2002; Aballay *et al.,* 2003; Couillault , 2004; Nicholas and Hodgkin, 2004) (Fig. 7).

The p38, JUN, and ERK MAP signal transduction pathways are homologous with mammalian signaling pathways

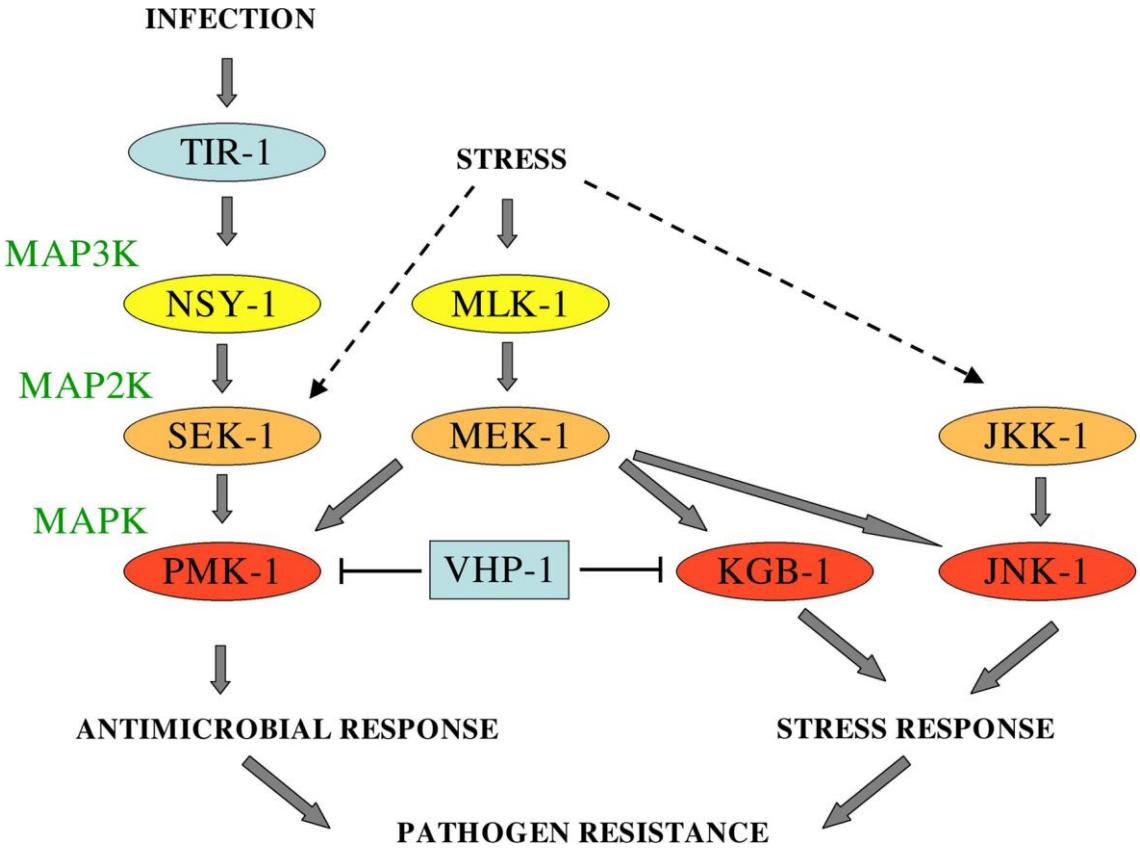

Fig. 7 A model signal transduction pathway for pathogen resistance in C. elegans (Ewbank, 2006)

Saccharomyces cerevisiae – Baker's Yeast

S. cerevisiae is not pathogenic to humans but in rare cases it is pathogenic to humans (McCusker *et al.*, 2006). *S. cerevisiae* cells produce a thick cell wall composed of proteins and polysaccharides which constitutes 25% of the cell's dry mass (Berry, 1982). Each cell is 2.5um-10um in diameter and oval in shape (Berry, 1986). The benefits associated with *S. cerevisiae* for the study of fungal pathogenesis are that its genome has been completely sequenced and all aspects of its biology have been extensively studied.

Fungal infections are difficult to treat, as both humans and yeast are eukaryotic, making it difficult to design anti-yeast drugs that are not toxic to humans (Heitman, 2005). In humans, fungal pathogens like *Candida albicans* are responsible for a disease called thrush (Gentles and La Touche, 1974). Though fungal pathogens to humans constitute only a small fraction of human diseases compared to bacterial and viral pathogens, in Mexico 50% of a population has been reported to be infected (Gentles and La Touche, 1974). In more economically developed countries, pathogenic fungi are found commonly in the form of the *Tinea* species that cause infections of the

nails and feet (Gentles and La Touche, 1974). Major threats of eukaryotic pathogens like fungi are of a great challenge as the biological machinery of fungi are very similar in nature to human cells (Heitman, 2005). Cases of invasive *Aspergillus* have been reported to have a high a mortality rate (66%) in patients who are transplant recipients (Nucci and Marr, 2005).

Methodology

Stock Plate Maintenance

All strains of worms were maintained at 20°C except for *fer-1* mutant worms which were incubated at 16°C. A previous grown *E. coli* OP50 stock cultured overnight in H-broth liquid media was spotted onto 60mm diameter NGM agar plates (Sulston and Hodgkin, 1988). These plates were used for all stock cultures.

Strains

Mutant *C. elegans* strains were obtained from the Caenorhabditis Genetics Center. The mutants, *mek-1 (ks54)* X*, mek-2n1989)* I*, fer-1 (b232)* I*, jnk-1 (gk7)* IV*, kgb-1 (km21)* IV*, pmk-1 (km25)* IV*, sek-1 (km4)* X*, nsy-1 (ag3)* II. Caenorhabditis Genetics Center. The wild-type strain used was N2, *C. elegans* var. Bristol.

Synchronous Worm Population

20 gravid hermaphrodites were transferred onto an *E. coli* OP50-spotted stock plate and were given 2-3 hours to lay eggs. After 100-120 eggs were laid, adult worms were picked off to produce a stock plate containing only eggs. Eggs were then incubated for 42h at 20C to develop into a synchronous population of L4/Adult molt larvae (Cass ada and Russell, 1975).

Experimental NGM Agar Plates containing Live E. coli, Heat Killed E. coli, and S. cerevisiae

E. coli OP50 were cultured on LB media (D'Ari, 2007) plates at 37°C and BY4742 mCherry yeast (a gift of Sherwin Chan and Gerald Fink) were cultured on YPD media plates (Rose *et al.,* 1990) at 32°. The BY4742 mCherry strain expresses RFP fused with

the promoter and coding sequence of the yeast pyruvate decarboxylase gene. Liquid cultures of *E. coli* were produced by picking a single colony into a 15mL sterile conical test tube with 5mL LB liquid media and incubating at 37°C overnight (Byerly *et al.*, 1976). Liquid culture of yeast was produced similarly with 5mL YPD liquid media and overnight incubation at 32°C. Heat killed *E. coli* OP50 was produced by immersing liquid-cultured *E. coli* in boiling water for 10 minutes.

Live *E. coli*, Heat Killed *E. coli*, and mixed Yeast/OP50 for test plate lawns were prepared by transferring 1mL of fresh liquid cultures to a 1.5mL eppendorf tube and pelleting the cells in a microcentrifuge for 5 minutes. The supernatant was removed via pipette and the cell pellet was weighed A working *E. coli* cell suspension of 200mg/mL was produced by adding the appropriate volume of dH20 and mixing to resuspend the cells. Similarly, a working yeast suspension was prepared to 20mg/mL cell suspension.

Each 60 mm test NGM plate was spotted with 2.5uL of 6.25mg/mL of *E. coli*, and 7.5uL of 1mg/mL of yeast, and 10uL of 50mg/mL streptomycin. For control plates *E. coli* alone, 7.5uL of dH20 was added instead of yeast. Spotted plates were incubated overnight at 20°C to dry.

Experimental culture started with L4 developmental stage worms

Twenty gravid hermaphrodites were transferred onto an *E. coli* OP50 spotted stock plate and were given 2-3 hours to lay eggs at 20°C. After 100-120 eggs were laid, adult parents were picked off to produce a stock plate with worm eggs. Eggs were then incubated for 42h at 20°C to allow development into a synchronous population of L4/Adult molt larvae (Cassada and Russell, 1975).

20 of these L4 worms were transferred to an experimental plate. This process was replicated with 5 plates to generate ~100 worms per experiment (Kelly and O'Brien, 2007). *E. coli* OP50 control plates were re-fed by spotting the plate with 20uL mixed solution composed of 2.5uL of *E. coli* (6.25mg/mL), 10uL streptomycin (50mg/mL), and 7.5 uL of H2O (Kelly and O'Brien, 2007). Yeast/OP50 plates were not re-fed by re-spotting as the yeast proliferated to a dense mass sufficient to provide an adequate food source.

Egg Preparation

A 1:4 ratio of bleach [~5% commercial sodium hydrochlorite (NaClO)] and dH2O was mixed with 1g NaOH to produce 100mL of alkaline sodium hydrochlorite solution (Stiernagle, 1999). A stock plate heavily populated with gravid adult

hermaphrodites was chosen for the egg preparation in order to obtain the maximum number of eggs. The stock plate was washed with 5mL of M9 buffer using a glass Pasteur pipette and the suspension of worms and eggs was transferred to a 15mL conical test tube (Brenner, 1974).

The worm/egg suspension was centrifuged on a table top centrifuge at 900 x g for a period of 1 minute. The supernatant was carefully removed to 0.5mL. 10mL of the alkaline bleach solution was added to the worm/egg suspension for a period of 2 minutes, during which the tube was mixed periodically by inversion, followed by an additional 1 minute of pelleting the worms via centrifuge. The supernatant was quickly removed and the worms were resuspended in 10mL of M9 buffer. This process of diluting out the bleach and washing the eggs was repeated 3 times.

The final egg suspension was made to 1mL with M9 buffer. Using a 100uL microcapillary glass pipette, a 10uL sample was spotted to determine the number of eggs present in 10uL egg suspension. The volume was adjusted to produce a 20 egg per plate experimental condition. A volume of egg suspension sufficient to give 20 eggs was spotted onto each experimental plate. Worms were observed after 3 days of incubation at 20°C, equivalent to the developmental age of adults one day past the L4-Adult molt.

Microscopy

Microscope slides were prepared as follows. A melted solution of 5% agarose solution containing 50uL 5% sodium azide as an anesthetic was dropped onto a microscope slide and quickly sandwiched by an addition of a second slide (Shaham, S. *ed.,* 2006). Slides were separated after the agarose hardened. 5uL of M9 buffer was dropped onto the agarose gel pad and 3 worms were transferred to the pad. The agarose pad was carefully covered with an 18mm square #1 cover slip (Sulston J. and Hodgkin J., 1988; Shaham, S. *ed.,* 2006).

A Zeiss Axio imager Z1 compound microscope equipped with a CCD camera, DIC optics, and epifluorescence was used in conjunction with the computer program AxioVision. Images were taken using the optical sectioning property of the Apotome imaging system. The “Low” Apotome grid was loaded and the noise reduction was set on medium with a ‘3’ setting. The green, red, and DIC channels were independently set to appropriate exposure times using the automatic exposure function. Images were taken with a 20x objective.

Images were edited using Adobe Photoshop CS3 to enhance contrast and to increase the intensity of color using the layer overlay tool. Images to be compared

were treated identically so as not to distort relative fluorescence intensities in different specimens. The pictures were photoshopped the same but sometimes the settings on the microscope were unnoticeably different and may have produced differences in background lighting and fluorescence intensity in some days.

Worm Recovery Experiment

L4 worms were grown on a mixed lawn of Yeast/OP50 for 48 hours at 20°C (Kelly and O'Brien, 2007). Worms were transferred onto an *E. coli* OP50 spotted stock plate for 10 minutes to clean off excess yeast. Cleaned worms were initially transferred onto *E. coli* spotted stock plates, and then transferred onto *E. coli* OP50 spotted experimental plates. Initial observations were made after 2h using microscopy.

Observational Analysis

Observations were carried out under the microscope at 200x final magnification using both DIC and epifluorescence. The degree of distension of the intestinal tract and the accumulation of *S. cerevisiae* were scored on a 4 point grading scale: none, low, mild, and severe. Scores were taken at 3 different regions of the worms' intestine, the posterior, mid-body, and anterior region.

An average of intestinal distension was calculated by taking the sum of the 3 regions of all the regions of all of the worms scored and dividing this figure by the total number of regions scored.

Lipofuscin intensity was scored on a 4 point grading scale: none, low, mild, and high. The relative lipofuscin intensity was based on the amount of overexposure the lipofuscin produced when GFP channel was set at 200 msec of exposure and examined at 20x objective.

An average of lipofuscin intensity was calculated by taking the sum of the 3 regions of all the regions of all worms scored and dividing this figure by the total number of regions scored.

The incidence of internal hatching of offspring was determined by the presence of hatched L1 larvae within the body of the worm. Examinations were made thoroughly by focusing up and down the focal plane of the worm viewed under DIC optics.

Colony Forming Unit Experiment with Silicon Carbide Beads

L4 worms were transferred to a mixed lawn of Yeast/OP50 plates and were

incubated for a period of 48 hours at 20°C. To preserve as much yeast accumulation as possible within the intestinal tract, the procedure was carried out at low temperature to reduce the rate of digestion and defecation. Worms were washed with pre-chilled M9 buffer and were transferred to a 15mL conical tube placed over crushed ice. To remove freely suspended yeast from the infected worms, the worm suspension was allowed to settle on the bench for a period of 3 minutes on ice. The supernatant was carefully removed and worms were resuspended with 10mL of pre-chilled M9 buffer. This process of diluting out excess freely suspended yeast was repeated 3 times.

A table top centrifuge was set to the lowest temperature setting. The worm suspension was centrifuged for 1 minute at 900 x g. The aliquot was removed and resuspended to 300uL with pre-chilled M9 buffer in a 4mL glass vial. 0.4g of 1mm silicon carbide beads (Biospec, Inc.) was added to the worm suspension and the worms were pulverized using a table top vortex for 1.5 minutes.

Serial dilutions of 100-, 500-, 1000-, and 5000-fold were made using M9 buffer. A 100uL suspension of pulverized worm mixture was spread onto YPD media plates using a sterile bent glass Pasteur pipette. The plates were incubated for 48 hours at 32°C. Colonies formed could readily be distinguished as *E. coli* or *S. cerevisiae* thus antibiotics were not added.

Table.1 A schematic table illustrating the various experimental dilution factors of worm mix

100uL + 900uL	**10-fold dilution**	**Spread 100uL= 100 fold dilution**
100uL + 900uL	**100-fold dilution**	**Spread 100ul= 1000 fold dilution**
100uL + 900uL	**1000-fold dilution**	**Spread 100uL=10,000-fold dilution**

Counting Colony Forming Units

Colony Forming Units (CFUs) were scored by counting the number of yeast colonies formed after 48h of incubation. CFUs per 100uL were calculated as the number of yeast colonies counted multiplied by the dilution factor spread onto the plate. Plates containing fewer than 30 colonies were discounted and plates containing a confluent lawn of colonies were also discounted. A plate was denoted UNC when there was an uncountable number of colony forming units.

Results

S. cerevisiae colonize the intestinal tract of C. elegans. *S. cerevisiae* colonized the intestinal tract of worms (Fig. 8D-G). In wild type, the degree of distension of the intestine gradually increased from none, low, to mild, as the time after plating on yeast increased from Day 1, 2, and 3 respectively (Fig. 8D, 8E, and 8F). On Day 4 of infection, S*. cerevisiae* accumulated to produce severe distension, causing the intestine to expand to almost the entire width of the worm (Fig. 8G).

Mek-1 and mek-2 mutants become severely distended with S. cerevisiae earlier than wild type. *Mek-1* and *mek-2* mutants exhibited aggressive *S. cerevisiae* accumulation within the intestinal lumen on Day 2 and Day 3 after plating on yeast, compared to wild type (Fig. 10A, Fig. 9D-F, and Fig. 11E-H). On Day 3, *mek-1* and *mek-2* developed severe distension, causing the intestine to expand to almost the entire width of the worm (Fig. 9F and Fig. 11G). Overall, yeast accumulation in the intestinal tract occurred more rapidly in the mutants than in wild type (Fig. 10A). Interestingly, the mek-2 mutant appeared to be more severely distended than mek-1 on all days tested.

S. cerevisiae infected mek-1 develop internally hatched offspring. In addition to *mek-1* and *mek-2* showing aggressive accumulation of yeast cells in the intestine, *mek-1* frequently developed internally hatched offspring when fed yeast (Table 2). This is reminiscent of the internally hatched offspring that are observed frequently in starved hermaphrodites. After 24hr of exposure to *S. cerevisiae, mek-1* presented internal hatching of offspring (Table 2). *Mek-2* also presented aggressive accumulation by *S. cerevisiae* but, did not develop internally hatched offspring.

Worms can recover from intestinal accumulation of S. cerevisiae **and intestinal distension.** *S. cerevisiae* infected controls for wild type and *mek-1* presented mild accumulation of *S. cerevisiae* on initial (2hr) and 24hr observations as expected (Fig. 12C-D and 12I). Recovered wild type and *mek-1* however did not present any trace of *S. cerevisiae* accumulation within the intestinal tract with accompanying very low distension of the intestinal lumen (Fig. 12E-F and 12J-K), suggesting that *S. cerevisiae* cells were completely defecated and or digested from the intestine after transfer onto an *E. coli* OP50 experimental plate. Recovered wild type and recovered *mek-1*, showed similar characteristics of healthy uninfected controls with respect to the observed low

intestinal distension of the lumen (Fig. 12A-B, 12E-F, 12G-H, and 12J-K). Recovery from intestinal infection by *S. cerevisiae* by transferring was also consistent in other MAP kinase mutants (Fig. 13).

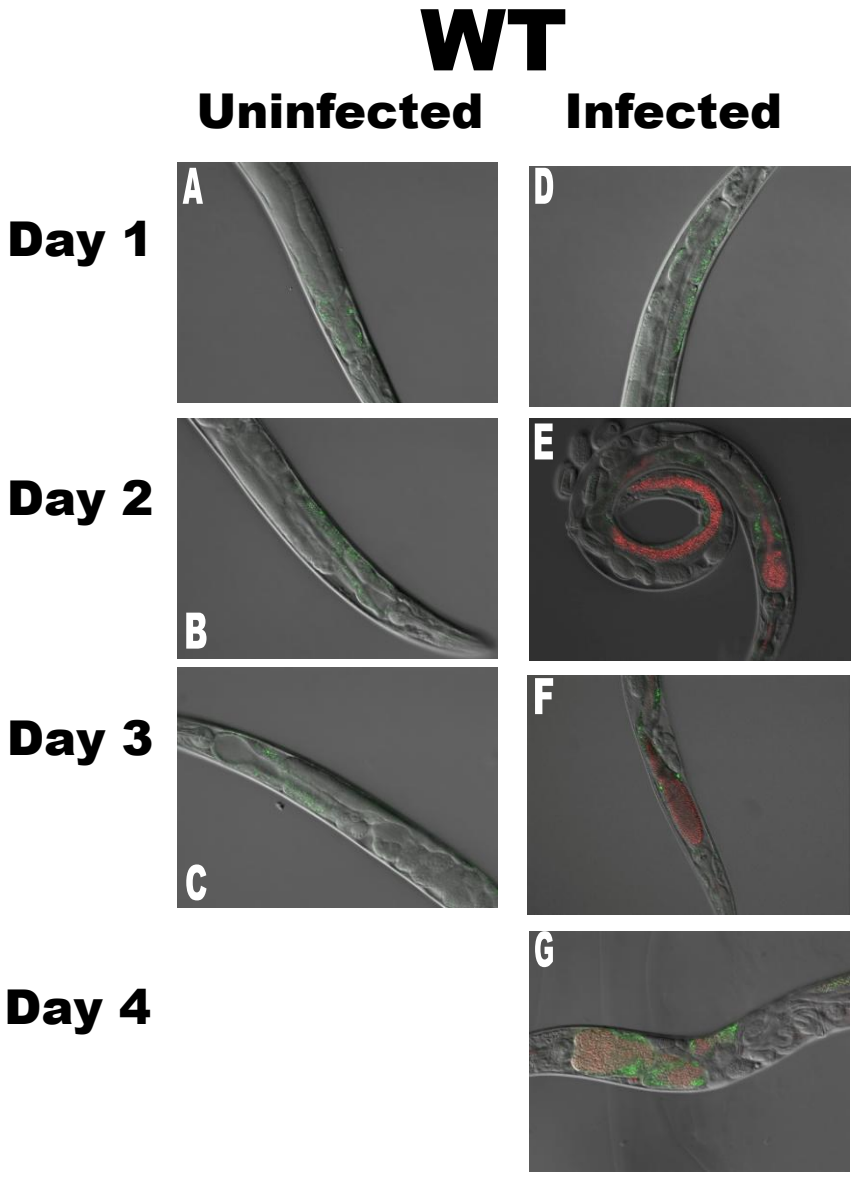

Fig. 8 *S. cerevisiae* accumulates within the intestinal tract and causes distension of the lumen in infected worms. L4 developmental stage worms were transferred onto experimental plates. **(A-C)** Uninfected wild type fed on a lawn of *E. coli* OP50. Intestinal lipofuscin auto-fluorescence (Green) is shown. **(D-G)** Infected wild type fed on a mixed lawn of *S. cerevisiae*/*E. coli* OP50. *S. cerevisiae* expressing red fluorescence protein (Red) and Intestinal lipofuscin auto-fluorescence (Green) are shown. Observations were taken every 24hr.

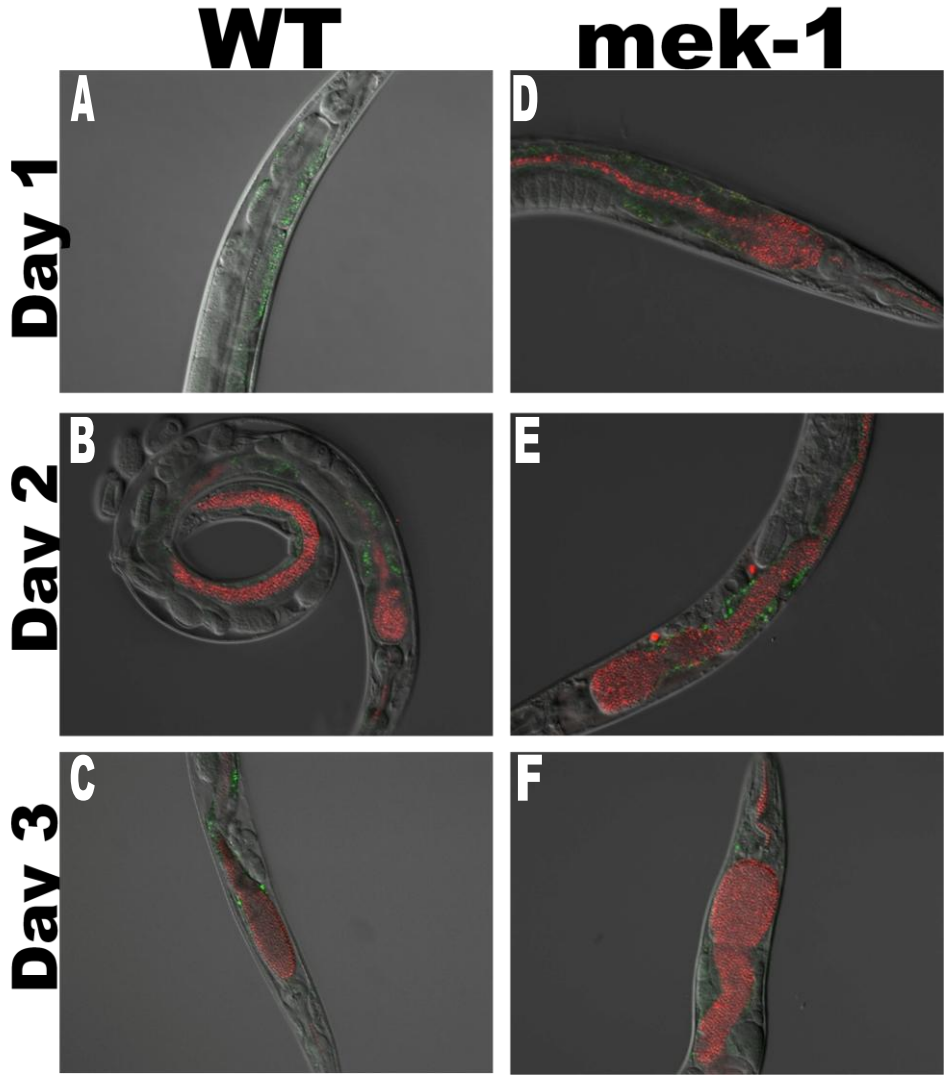

Fig. 9 *mek-1* mutants accumulate *S. cerevisiae* more rapidly and experience greater distension of the lumen than does wild type. L4 developmental stage worms were transferred onto experimental plates. ***(A-C)*** Wild type worms fed on a mixed lawn of *S. cerevisiae*/*E. coli* OP50 from the L4 stage. *S. cerevisiae* expressing red fluorescence protein (Red) and intestinal lipofuscin auto-fluorescence (Green) are shown. ***(D-F)*** mek-1 mutants fed on a mixed lawn of *S. cerevisiae*/*E. coli* OP50. Observations were taken every 24hr.

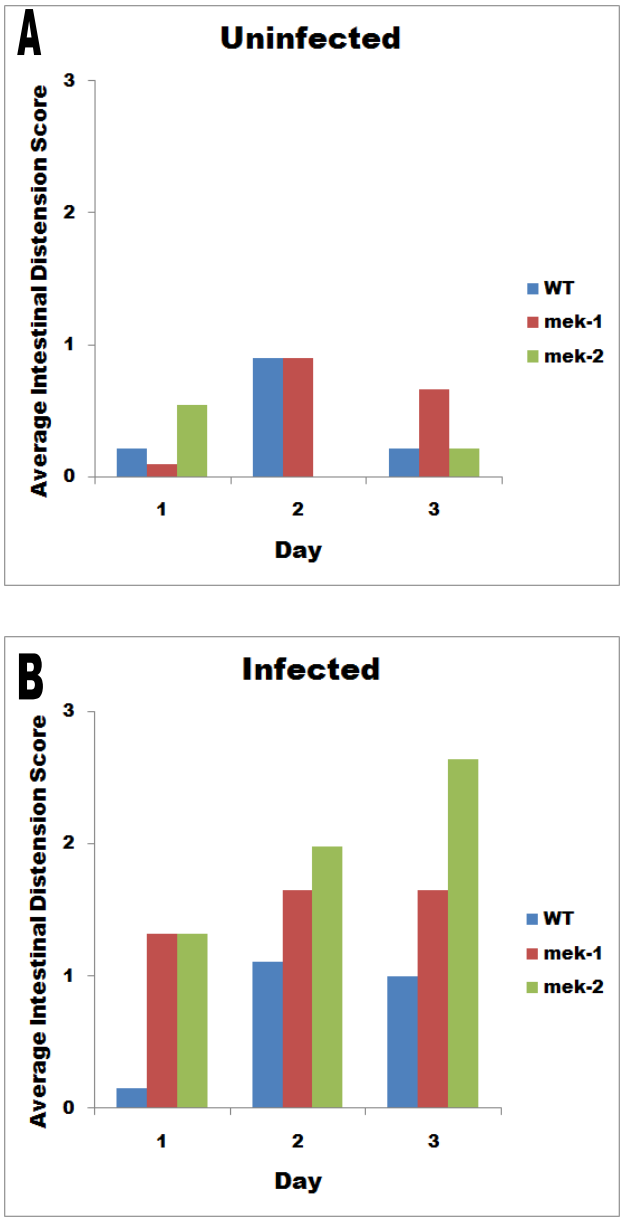

Fig.10 *mek-1 and mek-2* become highly distended with *S. cerevisiae* within the intestinal tract compared to wild type worms. L4 developmental stage worms were transferred onto experimental plates. **(A)** Uninfected wild type (Blue bar), *mek-1* (Red bar), and *mek-2* (Green bar), fed on a lawn of *E. coli* OP50. **(B)** Infected wild type (Blue bar), *mek-1* (Red bar), and *mek-2* (Green bar), fed on a mixed lawn of *S. cerevisiae/E. coli* OP50. Observations were taken every 24hr. The degree of intestinal distension was scored at 4 regions of the intestine and converted to an average.

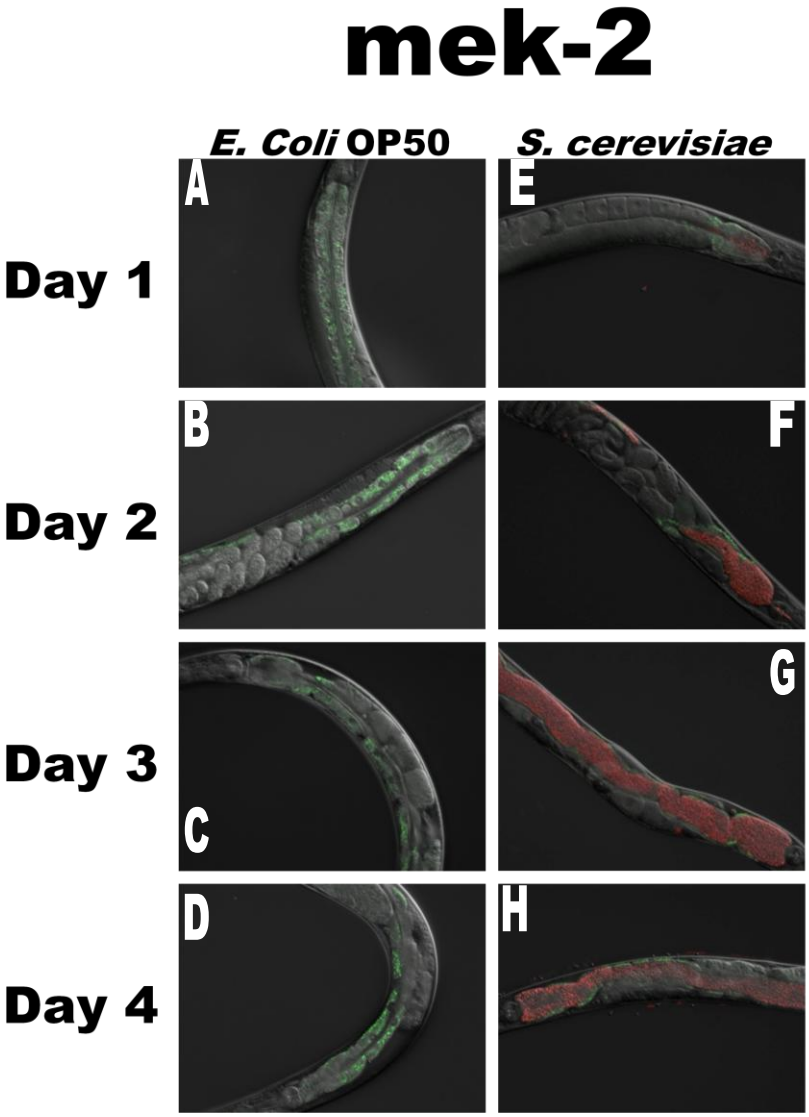

Fig. 11 *mek-2* mutant intestines become severely distended with *S. cerevisiae.* L4 developmental stage worms were transferred onto experimental plates. **(A-D)** *mek-2* fed on a lawn of *E. coli* OP50. Intestinal lipofuscin auto-fluorescence (Green) is shown. **(E-H)** *mek-2* fed on a mixed lawn of *S.* cerevisiae/E. *coli* OP50. *S. cerevisiae* BY4742 expressing red fluorescence protein (Red) is shown. Observations were taken every 24hr.

Table 2: Frequency of internal hatching of offspring in *S. cerevisiae* infected worms.

			No. of worms with internal hatching of offspring (%)			
			Uninfected Heat Kill OP50	Uninfected OP50	Infected *S. cerevisiae*	Recovered
Strain	Method	Day				
WT	L4[a]	1	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
		2	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)
		3	0/3 (0)	0/3 (0)	0/3 (0)	-
		4	0/3 (0)	0/3 (0)	0/3 (0)	-
	Egg[b]	1	-	0/3 (0)	0/3 (0)	0/3 (0)
		2	-	0/3 (0)	0/3 (0)	0/3 (0)
		3	-	0/3 (0)	0/3 (0)	-
		4	-	-	-	-
mek-1	L4[a]	1	0/3 (0)	0/3 (0)	0/3 (0)	3/3 (100)
		2	0/3 (0)	0/3 (0)	2/3 (66)	0/2 (0)
		3	-	-	-	-
		4	1/3 (33)	1/3 (33)	-	-
	Egg[b]	1	-	0/3 (0)	0/3 (0)	0/3 (0)
		2	-	0/3 (0)	2/3 (66)	0/3 (0)
		3	-	0/3 (0)	1/3 (33)	-
		4	-	-	-	-

[a]L4 developmental stage worms were transferred onto experimental plates

[b]Eggs were incubated onto experimental plates

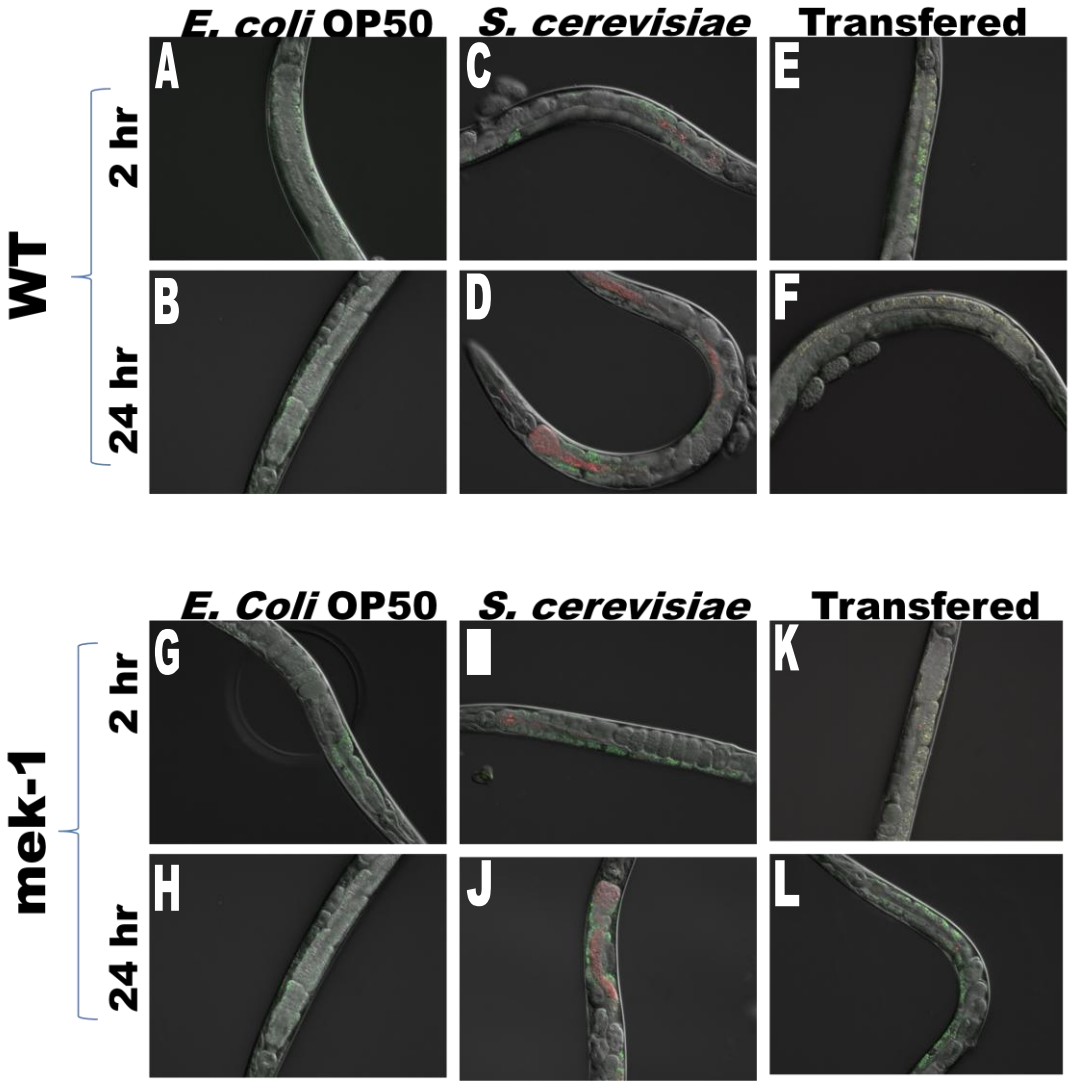

Fig. 12 *mek-1* worms grown on *S. cerevisiae* can recover after transfer onto *E. coli* OP50 lawn. L4 developmental stage worms were incubated under experimental conditions for 48hrs and observations were taken 2hr or 24hr later. **(A and B)** Wild type fed on a lawn of *E. coli* OP50. **(C and D)** Wild type fed on a mixed lawn of *S. cerevisiae*/OP50. *S. cerevisiae* expressing red fluorescence protein (Red) and lipofuscin auto-fluorescence (Green) is shown. **(E and F)** Wild type grown on a mixed lawn of *S. cerevisiae*/OP50 for 48hr and transferred onto *E. coli* OP50 lawn for recovery. **(G and H)** *mek-1* fed on a lawn of *E. coli* OP50. **(I)** *mek-1* fed on a mixed lawn of *S. cerevisiae*/*E. coli* OP5O. **(J and K)** Recovered *mek-1* transferred onto a lawn of *E. coli* OP50 for a period of 2h or 24h.

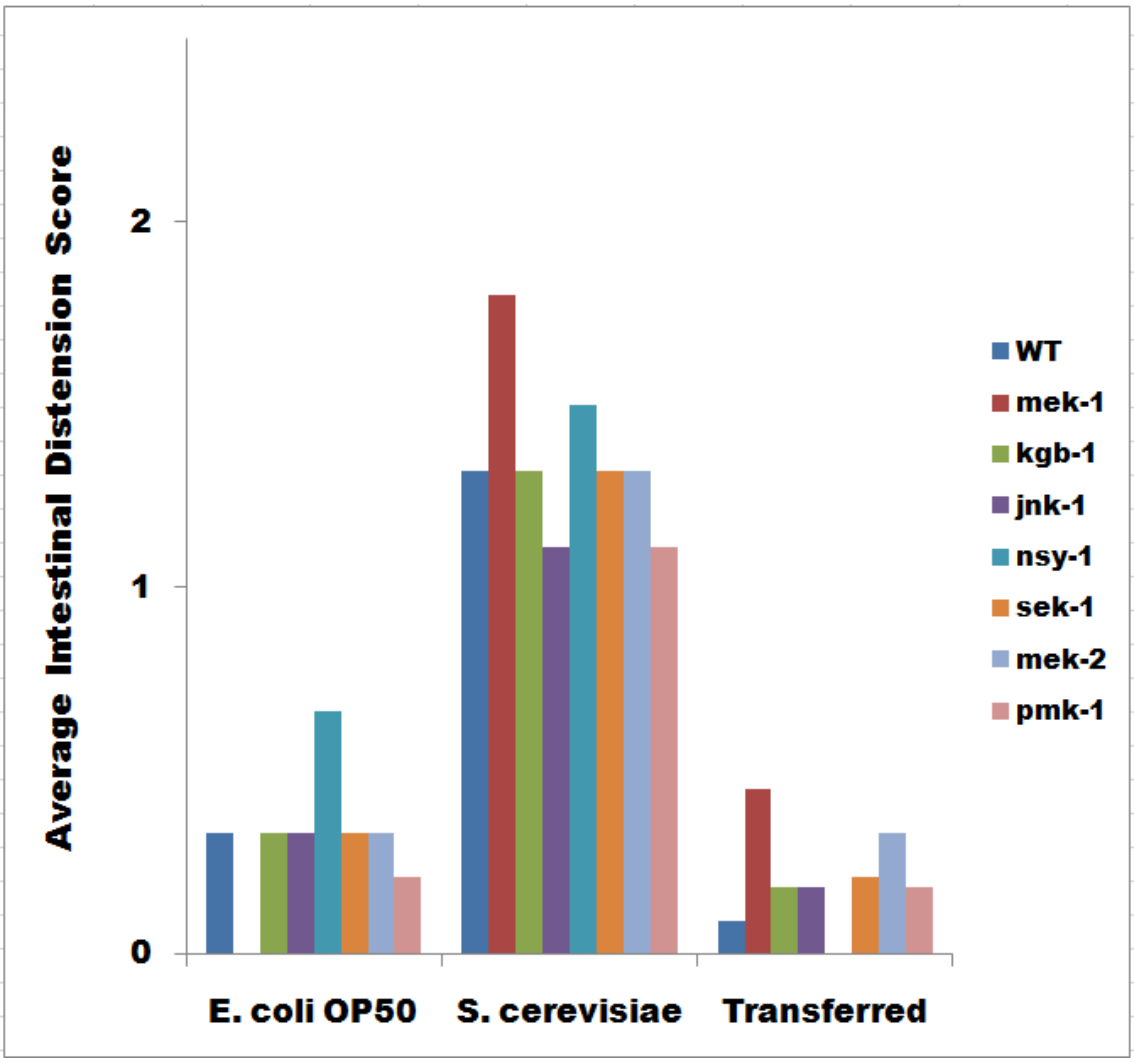

Fig. 13 Recovery of MAP kinase mutants from intestinal distension resulting from *S. cerevisiae* accumulation.L4 developmental stage worms were transferred onto experimental plates. Worms were grown on a mixed lawn of *S. cerevisiae*/*E. coli* OP50 for 48hr. Recovered worms were grown on *S. cerevisiae* for 48hr and were transferred onto a lawn of *E. coli* OP50. Uninfected worms were fed on a lawn of *E. coli* OP50 for 48hr. Observations were taken after 48hr of incubation. Intestinal distension of a single worm was scored at 3 regions of the intestine, then an average of 3 worms for that same particular day and strain was determined.

Growth on S. cerevisiae causes lipofuscin intensity to increase earlier. The intensity of auto-fluorescence was determined by the relative brightness and overexposure of fluorescence apparent when worms were excited for 200msec with the GFP channel. (Fig. 14D, 14E, 14F) The intensity of lipofuscin auto-fluorescence within the intestinal cells of infected worms was higher, compared to worms grown on lawns of *E. coli* OP50 and Heat Killed *E. coli OP*50. (Fig. 15)

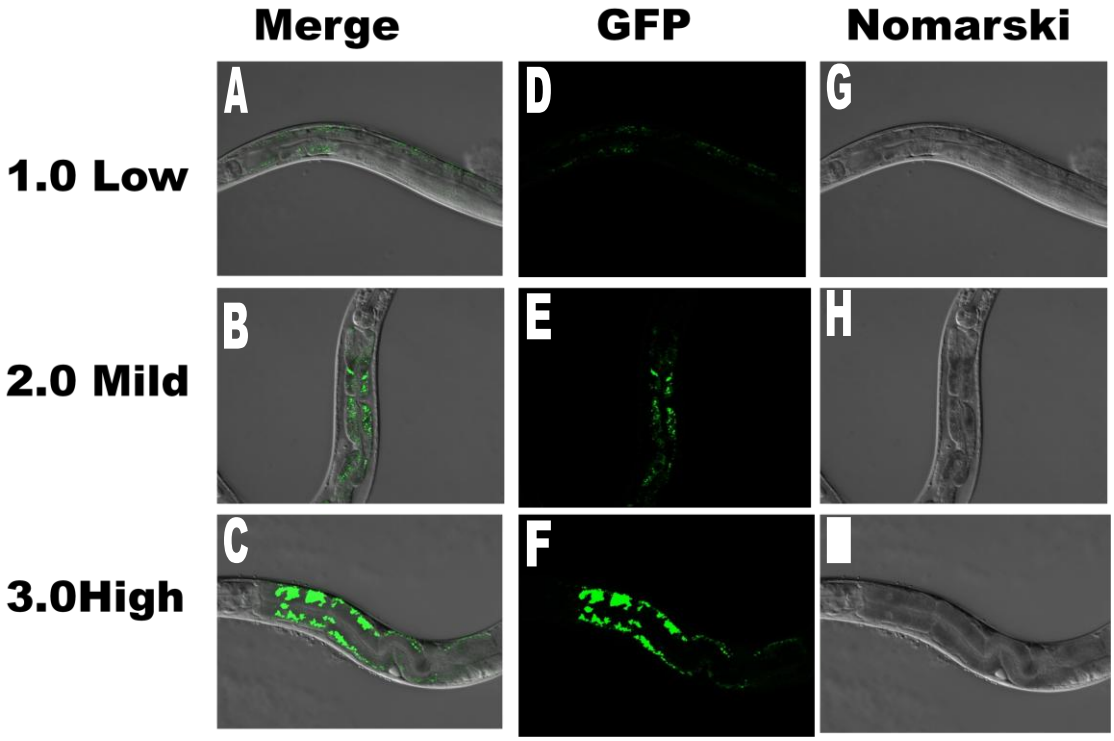

Fig. 14 Lipofuscin auto-fluorescence intensity was scored on a 3 point scale. (G-I) Intestinal cells of the worm were put into focus using DIC optics. **(D-F)** Lipofuscin auto-fluorescence images were taken with 200msec of exposure with GFP excitation. **(A-C)** Lipofuscin auto-fluorescence intensity was scored on a 3 point grading scale relative to the intensity of fluorescence; **(D)** low, **(E)** mild, and **(F)** high lipofuscin intensity. Observations were taken at 20x objective magnification.

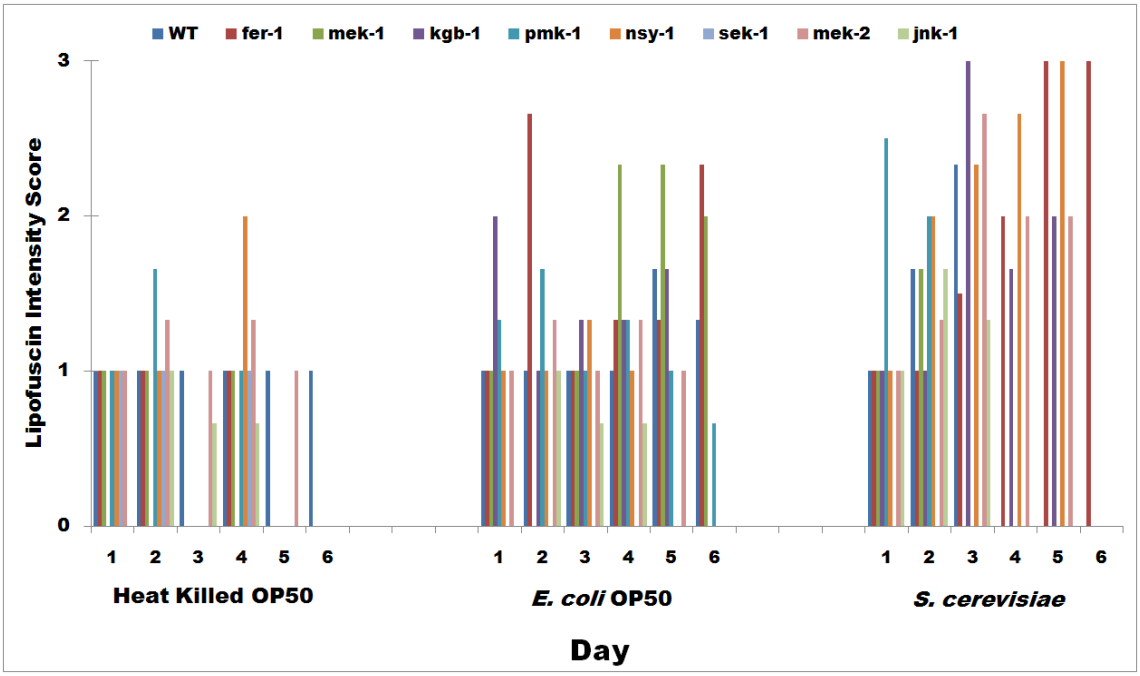

Fig. 15 Worms grown on *S. cerevisiae* develop increased lipofuscin auto-fluorescence intensity within the intestinal cells. L4 developmental stage worms were transferred onto experimental plates. Each strain was fed on lawns of either Heat Killed *E. coli* OP50 (Uninfected) or *E. coli* OP50 (Uninfected), or on a mixed lawn of *S. cerevisiae*/*E. coli* OP50. Lipofuscin auto-fluorescence intensity was examined by microscopy using the GFP channel with 20x objective. Lipofuscin auto-fluorescence intensity was scored on a 3 point grading scale relative to the fluorescence emitted with 200msec of excitation. (Fig. 14) Observations were taken every 24hr. Each bar corresponds to the average intensity of a particular day and strain.

Recovering worms by growing worms on yeast from egg.

Wild type and *mek-1* worms were grown on a mixed lawn of *S. cerevisiae* and *E. coli* OP50 from eggs as opposed to transferring worms onto experimental plates at the L4 developmental stage (Fig. 16). Both wild type and *mek-1* worms recovered from intestinal accumulation of *S. cerevisiae* after being transferred onto a clean *E. coli* OP50 plate (Fig. 17).

Kgb-1, pmk-1, jnk-1, and nsy-1, developed severe distension earlier than wild type. *Kgb-1, and jnk-1,* developed severe intestinal distension on Day 3 of infection similar to mek-1 and mek-2, 2 Days earlier than wild type. (Fig. 19 and Fig. 20).

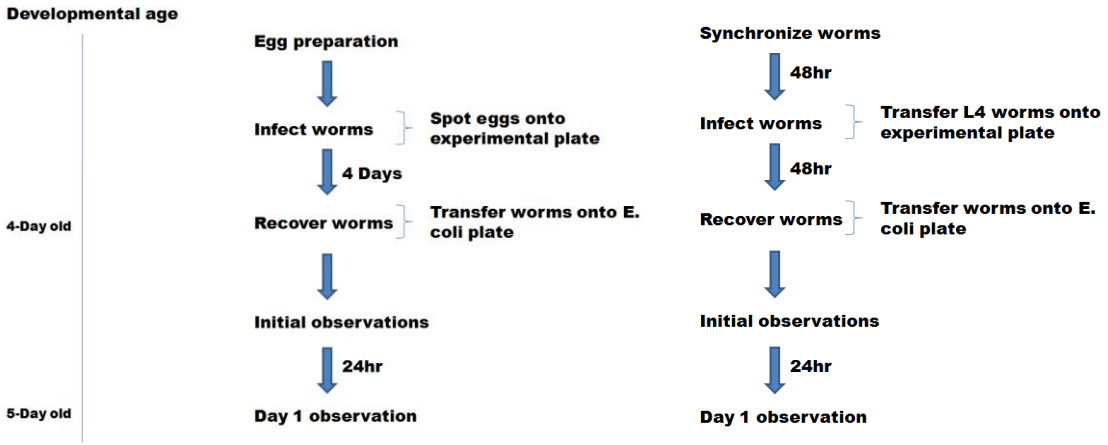

Fig. 16 Schematic for recovering worms by growing worms on yeast from egg versus L4 developmental stage

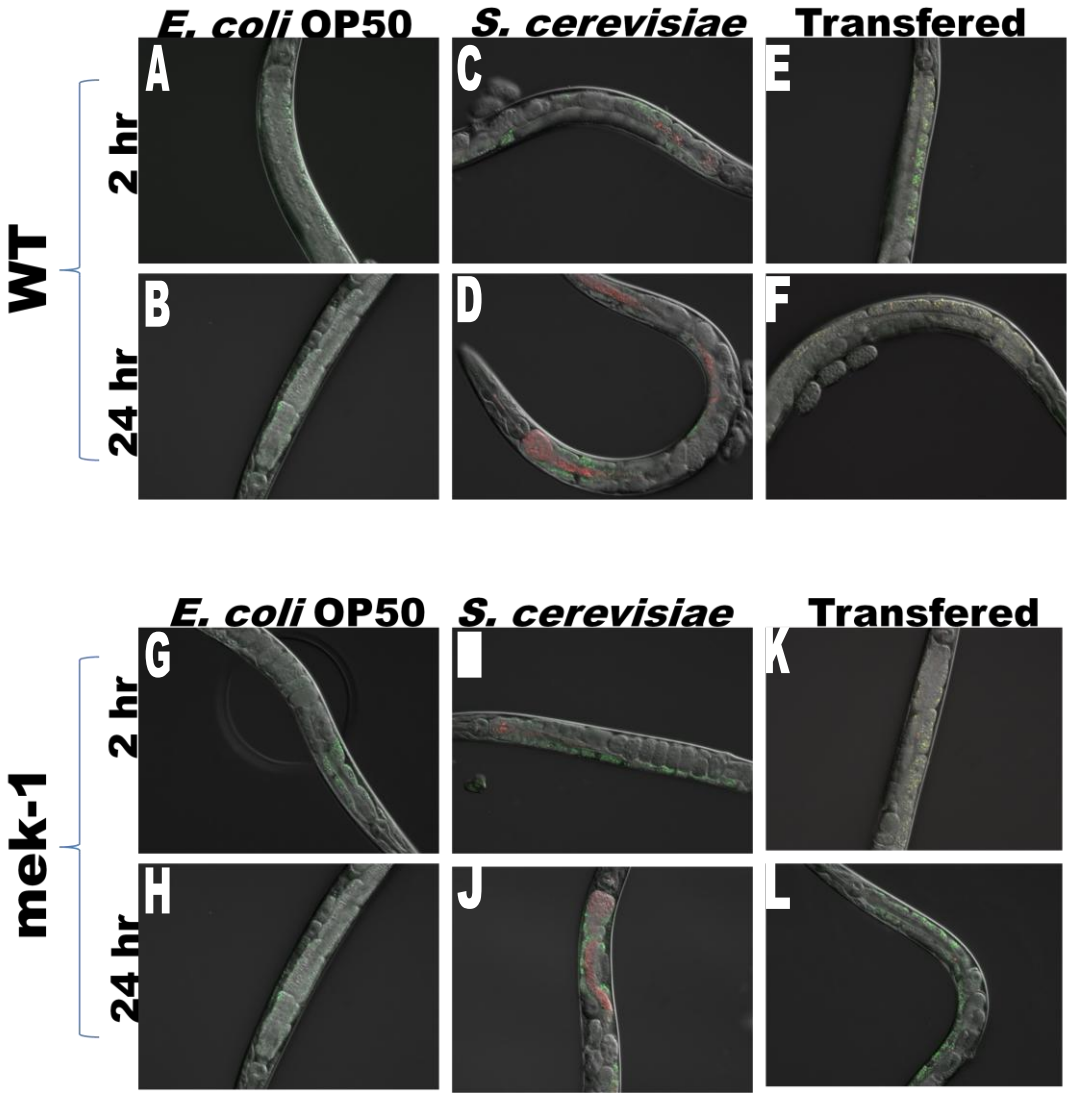

Fig. 17 Worms grown on *S. cerevisiae* from egg developmental stage can recover from after transfer onto *E. coli* OP50 lawn. Eggs were incubated on *S. cerevisiae* for 4-Days and observations were taken 2hr or 24hr later. **(A and B)** Wild type fed on a lawn of *E. coli* OP50. **(C and D)** Wild type fed on a mixed lawn of *S. cerevisiae*/OP50. *S. cerevisiae* expressing red fluorescent protein (Red) and lipofuscin auto-fluorescence (Green) is shown. **(E and F)** Recovered wild type transferred from a mixed lawn of *S. cerevisiae*/OP50 after 48hr onto *E. coli* OP50 lawn for recovery. **(G and H)** *mek-1* fed on OP50, **(I)** *mek-1* fed on a mixed lawn of *S. cerevisiae*/*E. coli* OP5O and **(J and K)** *mek-1* transferred from a mixed lawn of *S. cerevisiae*/OP50 after 48hr onto *E. coli* OP50 lawn for recovery.

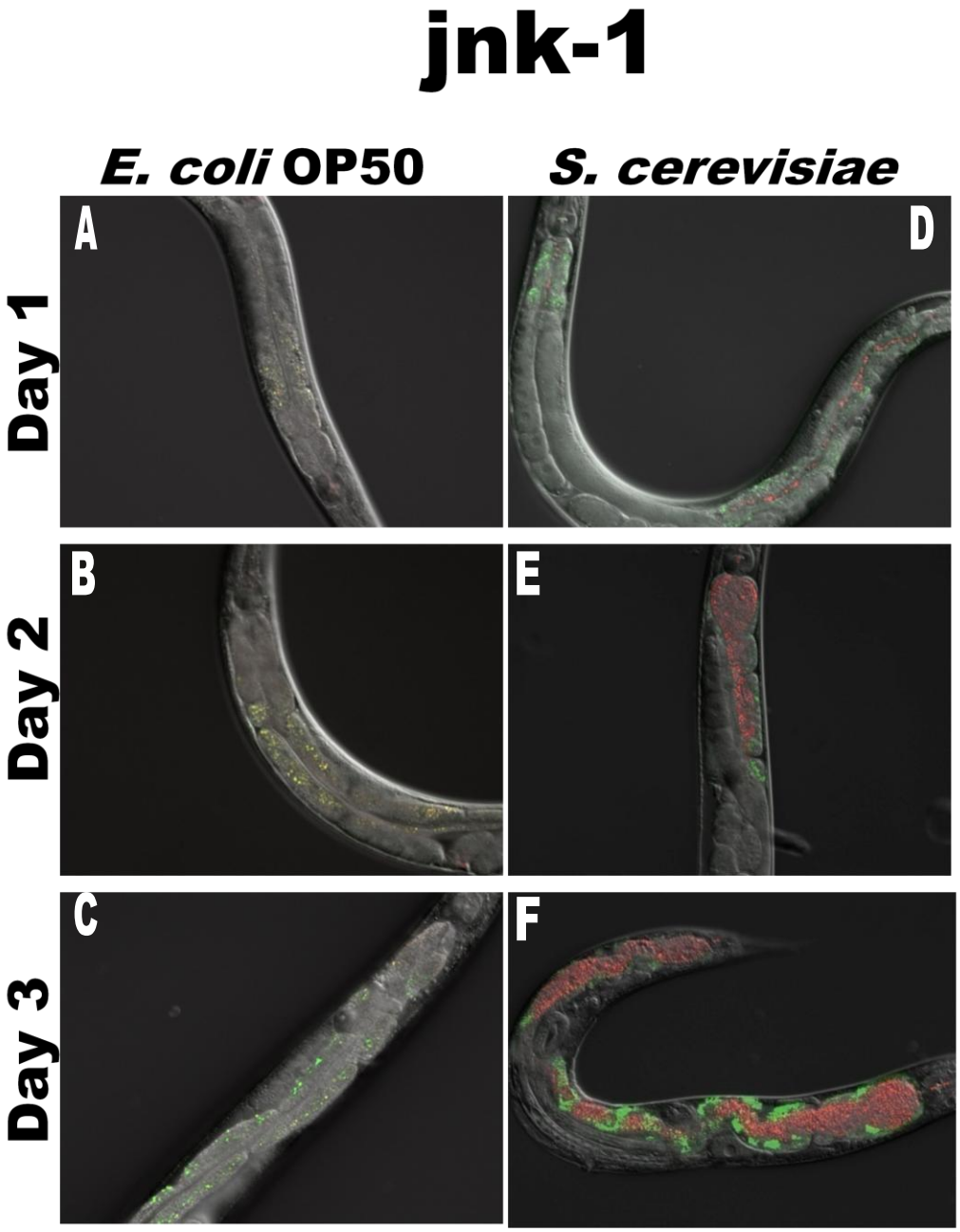

Fig. 18 *Jnk-1* mutant worms grown on *S. cerevisiae*. L4 developmental stage worms were transferred onto experimental plates. **(A-C)** *jnk-1* fed on a lawn of *E. coli* OP50. Intestinal lipofuscin auto-fluorescence (Green) is shown. **(D-F)** *jnk-1* fed on a mixed lawn of *S.* cerevisiae/E*. coli* OP50. *S. cerevisiae* BY4742 expressing red fluorescence protein (Red) is shown. Observations were taken every 24hr.

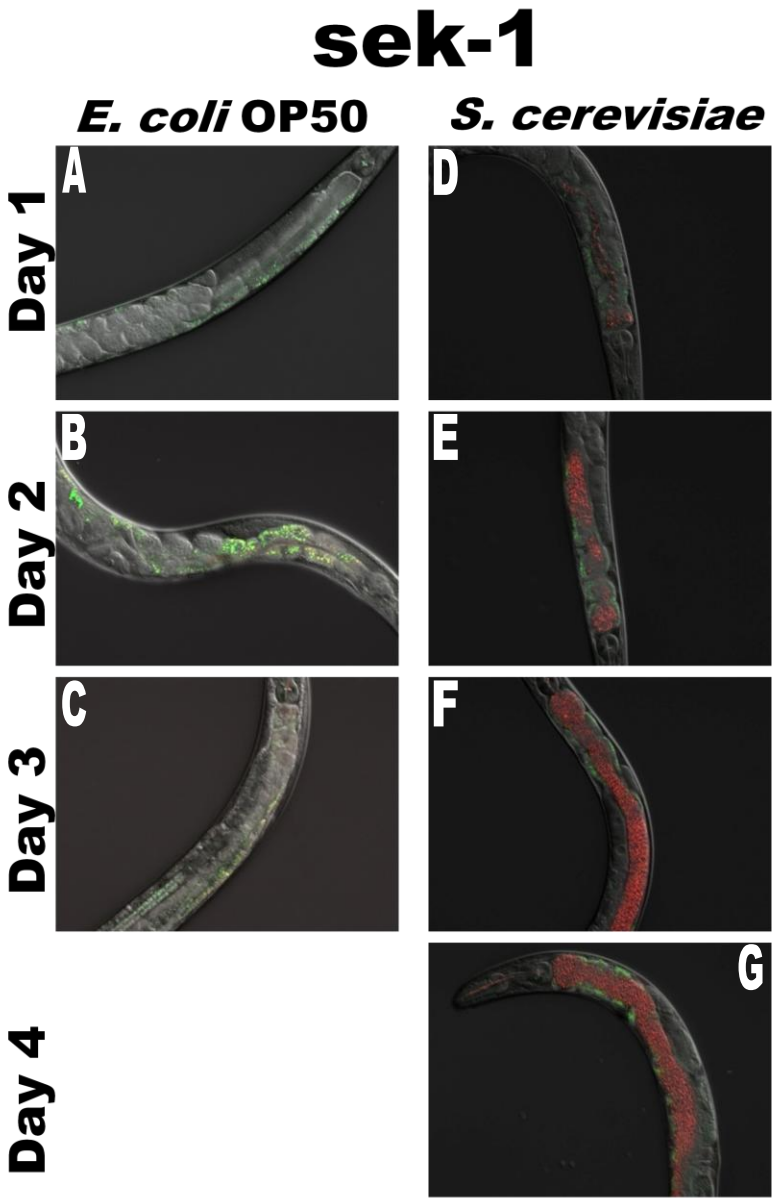

Fig. 19 *Sek-1* mutant worms grown on *S. cerevisiae*. L4 developmental stage worms were transferred onto experimental plates. **(A-C)** *sek-1* fed on a lawn of *E. coli* OP50. Intestinal lipofuscin auto-fluorescence (Green) is shown. **(D-G)** *sek-1* fed on a mixed lawn of *S.* cerevisiae/E. *coli* OP50. *S. cerevisiae* BY4742 expressing red fluorescence protein (Red) is shown. Observations were taken every 24hr.

Discussion

Mode of S. cerevisiae accumulation

C. elegans fed on *S. cerevisiae* accumulated *S. cerevisiae* within the intestinal tract (Fig. 9). The mode of accumulation appeared similar to infections of *C. elegans* by the Gram-positive bacteria *E. faecium* and *E. faecalis* (Garsin *et al.*, 2001; Kim *et al*., 2002)) and the fungus *Cryptococcus neoformans* (Mylonakis *et al.*, 2004). The degree of intestinal distension increased as the time of feeding on *S. cerevisiae* increased, eventually leading to severe distension (Fig. 9). Similar intestinal distension is caused by microbial infections and is also observed in *C. elegans* infected with *S. aureus* (Bae *et al.,* 2003; Begun *et al.,* 2005; Garsin *et al.,* 2001; Sifri *et al.,* 2003)

Mek-1 and mek-2 mutants become rapidly distended with S. cerevisiae

Mek-1 and *mek-2* mutants fed on *S. cerevisiae* developed a rapid intestinal accumulation of *S. cerevisiae (Fig. 9 and Fig. 11)*. *Mek-1* encodes a Mitogen-activated protein kinase kinase (MAPKK) which is thought to be active in the JUN Kinase pathway (Koga *et al.,* 2000). *Mek-1* is involved in stress responses (Koga *et al.,* 2000), starvation response (Koga *et al.,* 2000; Kondo *et al.,* 2005), heavy metal response (Koga *et al.,* 2000; Huffman *et al.,* 2004), and plays a part in the immune response (Koga *et al.,* 2000; Nicholas and Hodgkin, 2004; Aballay *et al.*, 2003; Kim *et al.*, 2004). *Mek-2* encodes a MAPK that functions as part of the ERK-mediated signal transduction pathway which regulates developmental processes (Wu *et al.,* 1995) and pathogen defense (Nicholas and Hodgkin, 2004).

Recovery from intestinal tract accumulation of S. cerevisiae.

Recovery from intestinal accumulation of *S. cerevisiae* was possible after transferring worms to a clean *E.* coli OP50 experimental plate (Fig. 12). This phenomenon of recovering from intestinal accumulation of pathogenic microbes is similar to *E. faecium* infections which do not persistently colonize the intestinal tract of *C. elegans* (Garsin *et al.*, 2001). However *E. faecalis* (Garsin *et al.,* 2001) and pathogens like the Gram-negative *S. typhimurium* establish a persistent intestinal infection (Ausubel *et al.,* 2000).

The recovery phenomenon suggests that the degree of accumulation of the intestine by *S. cerevisiae* is influenced by the rate of consumption and rate of excretion/digestion of *S. cerevisiae* cells. The rate of pharyngeal pumping and the rate

of defecation may have an important role during microbial infections of the intestinal tract, particularly with *S. cerevisiae*. The rate of pumping and defecation should be investigated in *C. elegans* carrying an intestinal accumulation of *S. cerevisiae*. In *Virbio vulnificus* infections the rate of pharyngeal pumping was observed to gradually decrease upon infection and an elongation of the defecation cycle was noted (Dhakal *et al.*, 2006).

Mek-1 was found to be expressed predominantly in the pharynx and intestine of worms, and has been implicated in regulating pharyngeal pumping in response to starvation (Koga *et al.*, 2000).

Correlated with *S. cerevisiae* infections, infected worms presented internal hatching of embryos leading to matricidal death (Table 2). It has been suggested that *S. aureus* infected worms become too weak to lay eggs, leading to matricidal death (Sifri *et al.,* 2003). In *E. faecalis* infections, diffusible toxins prevented infected worms from developing embryos; therefore internal hatching did not occur (Garsin *et al.,* 2001).

More lipofuscin intensity observed in worms fed S. cerevisiae

Increase in lipofuscin auto-fluorescence intensity was more pronounced in *S. cerevisiae* fed worms compared to both Live *E. coli* fed and Heat-killed *E. coli* fed worms *(Fig. 15).* In *E. faecalis* infections in *C. elegans,* the intensity of lipofuscin auto-fluorescence was distinctly stronger in worms fed *E. faecalis* than in worms fed *E. coli* (Chavez *et al.,* 2007). During an intestinal tract infection, ROS are produced which may combat the pathogen (Chavez *et al.,* 2007). Chavez *et al.* (2007) proposed a model of ROS secretion by intestinal cells which would be accompanied by superoxide dismutase (SOD) production to counteract the harmful effects of ROS to the intestinal cells (Chavez *et al.,* 2007). Increase in lipofuscin auto-fluorescence during an *E. faecalis* infection was suggested to result from the overproduction of ROS which leads to an accumulation of cellular damage in the form of lipofuscin (Chavez *et al.,* 2007).

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